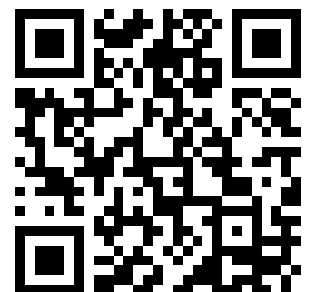

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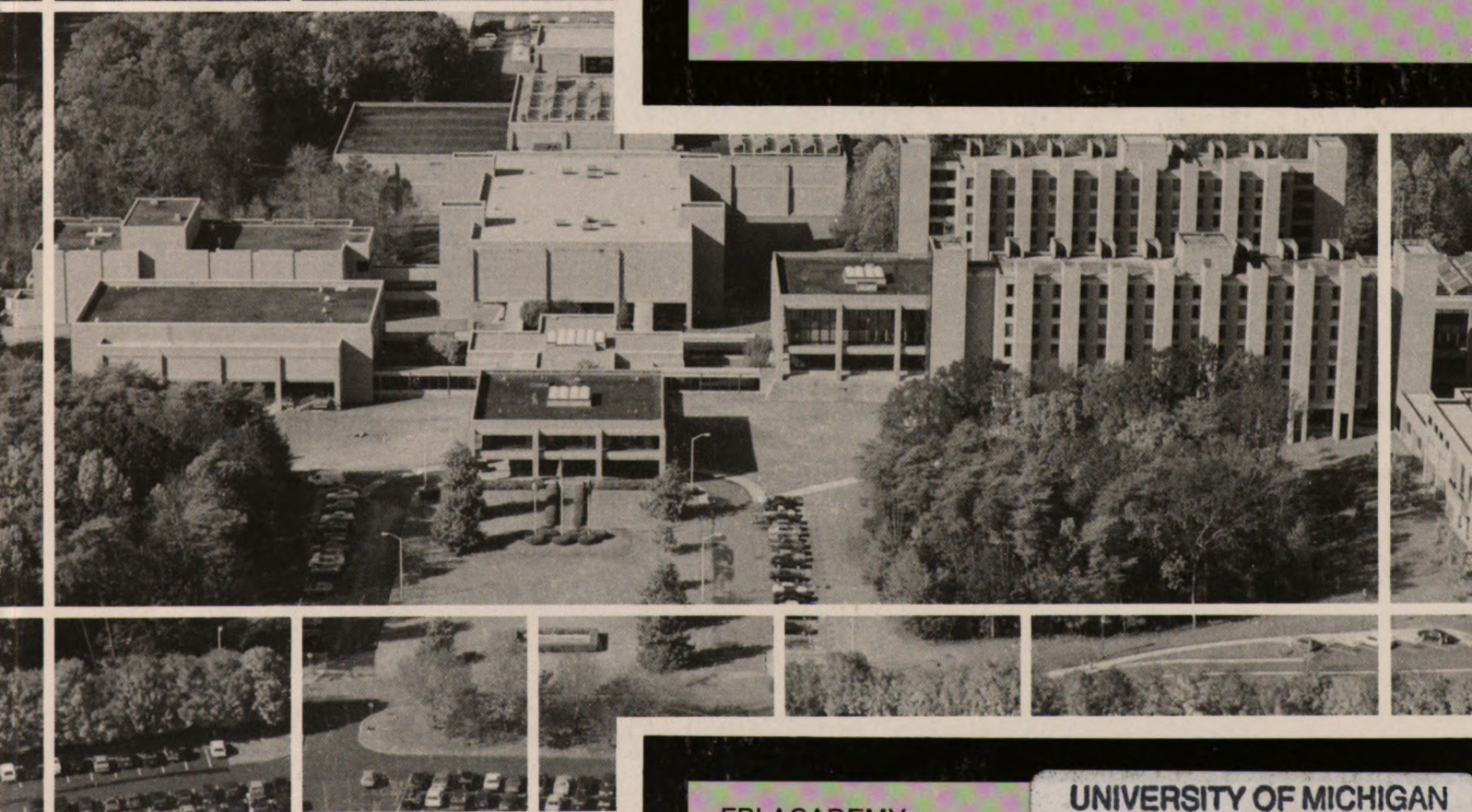
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PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM ON FORENSIC TOXICOLOGY



FBI ACADEMY
QUANTICO, VIRGINIA
JUNE 15-19, 1992

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FOREWORD

On June 15-19, 1992, the Federal Bureau of Investigation hosted an International Symposium on Forensic Toxicology. This symposium was conducted at the Forensic Science Research and Training Center (FSRTC) at Quantico, Virginia, and was attended by 180 scientists representing 150 laboratories from throughout the United States and 21 foreign laboratories.

This symposium brought together scientists from academia, commercial laboratories, and federal, state, and local medical examiner and crime laboratories to discuss issues associated with advanced technologies in forensic toxicology. This symposium, as with other previous forensic symposia hosted by the FSRTC, was considered a major success by those in attendance. Not only were new analytical approaches described, but also novel procedures were presented such as the use of robotics in analysis, analysis of drugs in hairs, analysis of chemical warfare metabolites, analysis of steroids, and the analysis of carrion-feeding insects in determining death by poisons, among others.

We hope that the spirit of cooperation and exchange demonstrated at this meeting continues, and that this publication serves as a useful reference for practitioners in this rapidly changing forensic discipline.

Assistant Director
FBI Laboratory
Washington, D. C.

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Section I

LECTURES

Forensic Toxicology: Where Do We Come From?

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When we define toxicology as the science of hazardous substances, it becomes clear from the early records of history that man has learned to live with hazardous substances for a very long time. The Ebers Papyrus of about 1500 B.C. already mentions a series of recognized poisons such as antimony; lead; copper; plants like hemlock, black nightshade, and spurge; as well as aconite, and opium. Not only was the emerging knowledge used for hunting and warfare, but also for a more rational medicine on the basis of antidotes. On the other hand, the utility of poisons was also rapidly recognized as a tool to remove undesirables from society.

Under the Greeks and Romans, murder by poison developed to such an extent that the leaders of those days had to take elaborate measures—such as using food tasters—in order not to become victims of arsenic, strychnine, cyanide, and the like. During the Middle Ages poisoning became an almost integral part of society and a political tool among “life at the top” especially in Italy and France. In those days, poisoning was almost seen as an art; experts in the field were in high demand and devices and methods of poisoning proliferated at an alarming rate. Very interestingly, women played a leading role in these developments as evidenced by such names as Toffana, Catharina de Medici, and Lucretia Borgia. In Rome there was a distinguished club that offered membership only to those ladies who had been able to effectively murder their husbands with poison.

With poisoning being so widespread, scientific knowledge also increased, of course. Attempts were made to establish the presence of poisons such as arsenic and lead, and the first legal autopsies for the investigation of poisoning cases were done in the early 1300s. Clearly the most important scientific figure in the Middle Ages

was Philippus Aureolus Theophrastus Bombastus von Hohenheim, also known as Paracelsus (1493 - 1541), who focused on the toxicon as a chemical entity and who initiated the basis of toxicology as a science by the Latin statement “*Dosis sola facit venenum*” (only the dose makes a poison).

BIRTH AND DEVELOPMENT OF FORENSIC TOXICOLOGY (1814 - 1945)

The years after Paracelsus saw a steady progress in the diagnosis and treatment of poisonings, but little attention was given to the chemical analysis of poisons in the body. It took until the early 19th century that the Spanish physician, Matthieu Joseph Bonaventura Orfila (1787 - 1853), working at the University of Paris, was successful in developing a scheme for chemical analysis for autopsy material and to combine the outcomes of these analytical findings with the pathological ones. In 1814 he published his classic book entitled “*Traité de Poisons ou Toxicologie Generale.*”

Orfila is seen by many as the founder of modern forensic toxicology. He had many followers such as Robert Christison (1797 - 1882), a professor of medical jurisprudence at the University of Edinburgh in Scotland; and Lewis Lewin (1854 - 1929), a leading professor of toxicology in Berlin.

The 19th century also brought many chemical developments thanks to such leaders as Runge, Von Liebig, and Fresenius and this sparked a tremendous interest in developing new analytical techniques also aimed at forensic toxicology. The first practical method of detecting arsenic was described by Marsh in 1836. Fresenius presented a cation separation procedure based on precipitations with H_2S in 1838; Reinsch developed

a method for arsenic and mercury in 1840. These methods had a great impact on the detection of criminal poisonings with the heavy metals and metalloids which were very much "en vogue" at that time.

Yet, also in the area of organic poisons there were important developments. After Sertürner had isolated morphine from opium in 1805, Christison came up with the ferric chloride/iodic acid test for morphine in 1845. In 1851 Jean Servais Stas, working in Brussels on an alleged case of nicotine poisoning, devised a scheme for the isolation of alkaloids from tissues by deproteinization with ethanol. This led to the well-known Stas-Otto method of 1856 that formed the basis for a systematic method of extraction of organic chemicals from tissues and body fluids.

These developments were of monumental importance for forensic toxicology. They introduced the principles of selective solubility/precipitation/extraction and thus laid the cornerstones for a rational approach to the isolation of potentially hazardous substances, followed by a subdivision in a variety of chemical classes (*e.g.*, acidic, basic, and neutral drugs) that is still being used today. Identification of the thus isolated substances/fractions was then attempted by techniques such as color reactions, crystal tests, melting points, emission of spectral bands, etc. This situation prevailed by and large until after World War II and the substances commonly involved in forensic toxicology also remained virtually the same: heavy metals, cyanide, carbon monoxide, alkaloids, and the first wave of potent synthetic drugs emanating from the pharmaceutical industry, such as barbiturates and analgesics. For more details on the history of forensic toxicology, the reader is referred to the overviews by Nyogi (1981), Doull and Bruce (1986), and Müller (1991).

FORENSIC TOXICOLOGY IN THE ERA OF TECHNOLOGY (1945 - PRESENT)

Analytical Techniques

After World War II, forensic toxicology changed drastically in many respects. The advent of modern technology resulted in a great number of novel instrumental techniques that proved extremely useful. Ultraviolet (UV) and infrared spectrometry became introduced in the late 1940s, later followed by fluorescence spectrometry, nuclear magnetic resonance, X-ray diffraction, and mass spectrometry, all providing

structural information on the substance(s) of interest. This was supplemented by dramatic developments in the area of separation techniques, first paper and thin layer chromatography, followed later by gas chromatography and high performance liquid chromatography. Not only were these techniques of importance to separate compounds, but they also proved to be extremely useful in the initial stages of detection and preliminary identification of potentially harmful substances. Moreover, when combined with appropriate detectors, the chromatographic techniques also serve as excellent quantitative tools.

For the analysis of inorganic substances various electrochemical techniques such as polarography and anodic stripping voltammetry were developed plus a variety of spectroscopic techniques such as flame and laser emission, atomic absorption, neutron activation, and electron microprobe.

In the area of isolation, the classical liquid-liquid extraction approaches remained relatively unchallenged for a long period of time. Yet, in the last decade there has been a strong advent of liquid-solid extraction approaches (or solid phase extractions) now that the manufacturers of these solid phases are capable of producing materials with sufficient batch-to-batch reproducibilities. An alternative of liquid-liquid extractions, namely the use of supercritical fluids (*e.g.*, liquid carbon dioxide) has become technically feasible, but is still in its infancy.

Last but not least, among the methodological developments are the immunoassays. They were introduced to analytical toxicology in the late 1960s in the form of radioimmunoassays and have since shown a staggering growth, not only with regard to types of immunoassays (based on enzyme reactions, magnetic spin, hemagglutination, fluorescence polarization, etc.), but also in their acceptance as a rapid and simple yes/no technique for the screening towards an ever increasing number of substances and substance classes at very low levels.

Toxicologically Relevant Substances

Another key factor with a decisive impact on forensic toxicology has been the boom in chemical synthesis after World War II, which resulted in hundreds of thousands of new compounds for a variety of activities in different economic areas. Quite a few of these new

compounds had potentially toxic properties, were produced in relatively large quantities, and became readily available. As a result, they soon became misused—accidentally or intentionally—and thus they invaded forensic toxicology. Among them were many pharmaceuticals, pesticides, herbicides, rodenticides, disinfectants, household products, steroids, etc. In addition, activities in the area of drug abuse and doping in sports emerged. All of these developments have broadened the number of relevant substances in forensic analysis tremendously; from a few hundred some decades ago to a few thousand at the present time. These problems are even further aggravated by factors such as metabolic conversions and the easy spreading of substances around the world, due to the abolition of trading and travel barriers. To exemplify this, the newest International Association of Forensic Toxicologists/German Research Society (IAFT/DFG) retention index database for gas chromatography contains some 6,500 entries (De Zeeuw *et al.* 1992c).

Scope of Forensic Toxicology

For a long time, forensic toxicology could be defined as the qualitative and quantitative analysis of toxicants in samples of human origin and their role and manner in causing death or near death (Stewart and Stolman 1961; Balkon *et al.* 1978).

However, in more recent years, there has been a tremendous proliferation into new areas such as human performance toxicology (including workplace toxicology and drug trafficking), forensic urine drug testing (*e.g.*, for drugs of abuse or doping in sports), environmental toxicology (in soil, water, and air), animal toxicology, food toxicology, warfare toxicology, etc. Thus, the emphasis is no longer on poisonings in man, but now focuses also on man and its environment, the short- and long-term impact of chemicals on the quality of life, and on curbing unwanted social habits. Therefore, a modern definition of forensic toxicology appears to be the qualitative and quantitative analysis of potentially harmful substances in relevant specimens and the interpretation of the results in a given legal context or towards restrictive regulations.

The advent of these new areas has widened the breadth of forensic toxicology dramatically with regard to species, types of specimens, number of substances to be considered, interpretation issues, etc. Also, the types and numbers of analyses required nowadays has grown

enormously. On the other hand, it should be realized that a sizable percentage of the human population is now being subjected to some kind of forensic toxicology testing with considerable implications. All of this makes it essential that the forensic toxicologist is adequately trained and experienced, that he uses approaches and methodologies that are legally defensible, and that his results and interpretations are scientifically correct and undisputable.

WHERE DO WE STAND TODAY?

Fortunately, the developments outlined above have not remained unanswered. Forensic toxicologists around the world have spent tremendous dedication, time, and effort to meet the newly imposed challenges. As a result, one can conclude that much has already been accomplished, but it is also realistic to say that a lot still remains to be done. Moreover, newer developments and challenges are coming our way in rapid succession and increasing complexity.

It is good and timely, therefore, that this symposium is being held. It provides a much-needed opportunity to review the state of the art in modern forensic toxicology and to define our aims for the future. To this end, the organizers have been able to attract a large number of outstanding authorities to share their views with us and I would not dare to impinge on their areas of expertise. However, there are two general problems in qualitative analysis that I would like to address in more detail, namely systematic toxicological analysis and correct substance identification. These interrelated issues are key factors in forensic toxicology, yet they are often being neglected and/or misunderstood.

Systematic Toxicological Analysis (STA)

This area comprises the logical chemical-analytical search for a harmful substance whose presence is unsuspected and whose identity is unknown. Obviously, it applies to the so-called general unknown cases, but it should be stressed that STA is an essential part of a much broader range of cases, including those where at least one substance is known—or presumed to be—involved. Apart from detecting and identifying specific chemical substances, the analyst must also establish the absence of other potentially harmful substances in a given case (Cravey and Baselt 1981). The opposite of STA is the directed analysis which aims at one or more specific substances or classes of substances (*e.g.*, ethanol

in blood, opiates in urine). The latter area has seen tremendous developments in recent years with regard to sensitivity, selectivity, simplicity, and speed to the extent that we hardly realize anymore what kind of a miracle we perform if, for example, we correctly analyze a picogram of a substance in a milliliter of whole blood. Unfortunately, the situation in STA is far less favorable. Curry (1976) already noted that progress in STA since World War II had been rather limited and although there have been some notable developments since, the analyst involved in STA still faces a most difficult task because of the following complicating factors:

- a) The wide variety of compounds and the vast numbers that are to be taken into account.
- b) The relatively low levels at which they occur in a complex matrix.
- c) The lack of time to carry out in-depth analyses.
- d) Insufficient knowledge about the potentials of the analytical techniques used and, consequently, about the validity of the findings.

With regard to techniques, the general situation for the analysis of organic substances is that the analyst has a series of analytical methods of his own choice that he can apply, usually consisting of a series of color reactions, chromatographic techniques [thin-layer chromatography (TLC), gas-layer chromatography (GLC), high-performance liquid chromatography (HPLC)], spectrometry [UV, mass spectrometry (MS)], and an array of immunoassays. These methods are sometimes applicable directly to the specimen, but in most cases some kind of extraction or cleanup has to be done first. Yet, how to make sure that all relevant substances are detected and once detected, how to identify them properly against a background of thousands of others? In recent years, we have developed the following approach to STA (Schepers *et al.* 1983; De Zeeuw and Franke 1990; Franke and De Zeeuw 1991):

1. Evaluate the various analytical techniques and systems and express their value towards STA in terms of an objective scientific criterion such as their Identification Power (IP). Do this for single systems as well as combinations of systems.

2. Select the best systems available for STA considering their IP and practical applicability (costs, throughput, etc.) and designate these as recommended systems.
3. Collect reference data for as many as possible toxicologically relevant substances in these recommended systems (*e.g.*, Rf-values, retention indices, UV spectra, mass spectra) and set up databases for interlaboratory use.
4. Analyze a case by subjecting the specimens to the recommended methods and compare the results for the unknown(s) with the reference values in the (computerized) databases.

It should be noted that the above tasks 1 - 3 are far too large for an individual laboratory. Moreover, it would be a waste of time and effort. Instead, it should be carried out as a joint project by a consortium of qualified and interested laboratories around the world, leading to comprehensive databases that can be utilized at an interlaboratory level.

The above approach has since been taken up by the STA Committee of the IAFI, in cooperation with the Senate Commission on Clinical Toxicological Analysis of the DFG. In the meantime, this has led to recommended systems and databases for GLC and TLC (Ardrey *et al.* 1985; Moffat *et al.* 1987). Expanded databases are in preparation and will appear later this year. Recommendations and databases for other techniques such as HPLC and MS are in progress (De Zeeuw *et al.* 1992a; 1992b; 1992c). Also, the potentials and problems of extraction/sample work-up in STA are now being considered. Thus, in recent years there has been considerable progress in our understanding of how STA should be approached. Although it represents a tremendous analytical problem, we have come to appreciate the potentials as well as the constraints of the various techniques that can be used. Furthermore, the need for adequate documentation and access to computerized data storage and retrieval for substance identification has become evident (Franke *et al.* 1985). However, a lot still remains to be done. Newer techniques need to be evaluated and, when accepted, reference data are to be generated. In addition, the constant updating of the databases with new substances is an important task, though not very attractive. Yet, it should be stressed that a substance will not be found (or it will be misidentified) if it is not present in the

databases. Also, there is a tendency to limit the testing only to those substances that are commonly seen in forensic toxicology. Those to which this may apply should read Alan Curry's excellent letter in the IAFB Bulletin (Curry 1992). Finally, it is obvious that further advancements in STA require concerted, international efforts from different laboratories around the world. This will require financing on an international level, yet organizations willing to sponsor such endeavors are still to be found.

Substance Identification

The final step, both in directed searches and in STA, is the unequivocal identification of the substance(s) present. Here, the general belief seems to be that this can be achieved by a two-test approach:

- I. An initial or screening test, which is then translated into a substance candidate that is presumed to be present; and
- II. A confirmatory test based on a different physical or chemical principle from that of the initial test, if possible by MS.

This approach can be found in various guidelines, such as the National Institute of Drug Abuse Mandatory Guidelines for Federal Workplace Drug Testing Programs (1988), the Society of Forensic Toxicology/American Academy of Forensic Sciences Laboratory Guidelines (1991), and the European Community Directive for Residues in Food (Commission of the European Communities 1989). Unfortunately, such an approach has a number of serious shortcomings and cannot be considered scientifically correct:

- It hinges to a very large extent on the initial test. If the latter is not chosen properly, a false negative will result. In addition, when STA is involved, it is unrealistic to assume that one initial test would suffice.
- The ultimate aim of qualitative analyses is to exclude the presence of all (relevant) substances, except one. Again, this requires a multitude of tests, rather than a single initial test.
- The term "confirmatory test" implies that a presumption exists after the initial test. However, if this presumption is not correct, the

confirmation will be negative.

- A positive confirmation only means that its results are not against the initial presumption. Yet, it does not rule out other substances that may give the same results.

The above drawbacks become more severe when the spectrum of relevant substances increases. Therefore, a better way to correct qualitative analysis in forensic toxicology would be a two-phase approach instead of a two-test approach:

- I. Screening phase, consisting of a multitude of different tests, run parallel, to find specimens that give some kind of positive response.
- II. Identification/exclusion phase, additional tests to provide unequivocal identification of the substance(s) present, at the same time excluding all other relevant compounds.

At present, at least for the bulk of organic substances, the most rational screening techniques are immunoassays and chromatographic techniques such as TLC, GLC, and HPLC with suitable detection modes. The screening tests are to be chosen and combined in such a way that as many as possible relevant substances be detected. Obviously, what is to be considered relevant depends on the scope of the analysis.

Specimens that test positive in one or more Phase I tests are then to be run in Phase II to provide full identification/exclusion. Also, it must be borne in mind that many forensic toxicology laboratories around the world have no MS available. MS will of course be a powerful tool in Phase II, but it is incorrect to believe that MS is an absolute necessity. A proper combination of Phase I and Phase II tests may be equally suitable, provided that the validity of the various tests is adequately documented. However, the latter also applies to MS. It will be clear that the basic requirements for the two-phase approach are the proper evaluation and standardization of the analytical techniques for Phases I and II and the development of suitable databases for substance documentation and retrieval. As outlined above, progress is being made in these areas, albeit slowly. For a variety of analytical techniques their utility towards STA has been assessed and computerized databases for these techniques are becoming available. This will allow the analyst to choose from the analytical facilities and

instrumentation that he has available and to use his personal computer for rapid, unbiased substance identification. So far, two systems based on this approach have become commercially available (Toxi-Lab 1990; Merck 1992) and extensions can be expected in the near future.

In conclusion, forensic toxicology is changing and growing at an astounding pace. In order to be able to respond properly to the various challenges and to the ever-increasing demands that we are facing, we need to carefully consider what our strengths and weaknesses are and to define our aims and goals for the future. I am convinced that this symposium can play a key role towards meeting these objectives.

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Current Approaches to Driving Under the Influence Testing

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Decreasing societal tolerance of chemical-induced impaired driving together with improving analytical capabilities have prompted many new driving under the influence of drugs (DUID) laws in recent years. Many of the prosecutions resulting from these laws rely heavily on qualitative or quantitative corroboration of drugs in body fluids of those charged.

While much progress has been made in DUID testing, methodologies are still evolving. Certainly improvements in gas chromatography, high performance liquid chromatography, and mass spectrometry have extended the abilities of DUID laboratories in recent years. Arguably however, the availability of sensitive immunoassays for preliminary, presumptive testing has made the greatest contribution toward improved efficiency and cost effectiveness in DUID testing.

Presently immunoassays are the most widely used initial approach to detecting drugs in individuals charged with impaired driving. Fluorescence polarized, enzyme multiplied, and radioimmunoassays have been used with increased utility in recent years. Many of these proprietary immunoassays that were originally marketed only for use with urine have now been applied successfully to hemolyzed whole blood as well. Often little or no preliminary treatment of blood is necessary, thus expanding their utility to either sample for DUID testing. All immunoassays have the advantages of small sample volume requirements, ease of automation, and low incidence of false results, especially false negative results.

As a general rule, if a particular immunoassay response is negative (*i.e.*, below a detection threshold) additional confirmation of the negative result is usually unnecessary. However, the definition of any particular detection threshold will often vary from laboratory to

laboratory or even from purpose to purpose within a laboratory. Numerous factors influence the selection of those thresholds. Statutory or regulatory definitions of target drug concentrations obviously influence the selection of detection thresholds. If minimum reportable drug concentrations are defined by law or regulation, detection thresholds are set accordingly. Also, pharmacological or toxicological correlations between drug concentrations and effects on driving, if known, influence the selection of detection thresholds. Usually there is no need to extend analytical sensitivity below that where toxicological significance begins. However, one arguable exception exists when the active drug concentration in blood may decline substantially in the time interval between the offense and sampling (*e.g.*, as with cocaine or delta-9-tetrahydrocannabinol). Still other factors influencing a detection threshold include matrix effects and the inherent sensitivity of the immunoassay versus that of the confirmatory test. All of these factors are further impacted by the fundamental intent of the toxicological testing.

Obviously, the intent of DUID testing is to detect potentially impairing drugs, many of which require state-of-the-art screening and confirmation sensitivity. However, different rationales for drug screening may promote the use of different screening (*i.e.*, immunoassay, detection thresholds). One rationale is to consider immunoassay testing as a "gatekeeper." With this approach the immunoassay merely eliminates samples that are clearly negative from further testing. Only the subsequent analysis (or preferably analyses) define which sample is positive and which is negative. Here the immunoassay result is not considered a part of the confirmation process and a lower immunoassay threshold is justified. Any sample with an immunoassay response discernibly above "noise" might be included with those submitted for further testing. The only consequence of this liberally defined immunoassay

threshold would be some loss in efficiency in the subsequent, definitive analyses.

In the second rationale the immunoassay result is considered to be an independent and necessary part of the verification of a positive result. Using this rationale, the initial (positive) immunoassay test must be able to withstand adversarial scrutiny on its own. Here a more conservative definition of an immunoassay threshold is necessary. Some would believe that it is not justifiable to issue a positive DUID report where an equivocal immunoassay result is paired with a positive confirmation result, even if that confirmation technique were gas chromatography/mass spectrometry (GC/MS).

The avoidance of both false negative reports and, especially, false positive reports is important in DUID testing. Consequently, if immunoassays are an integral part of the verification of positive results, defining the immunoassay detection threshold is as important as defining GC/MS confirmation criteria. Using immunoassays as such, a conservative definition of this threshold becomes necessary. With an empirical approach the immunoassay detection threshold may be defined as the immunoassay response above which confirmation is "probable." This involves establishing the correlation between immunoassay response and the probability of GC/MS confirmation with actual samples. This correlation will likely vary between laboratories for any given numerical immunoassay response and may vary over time within any one laboratory as well. For example, one laboratory may be able to document a 90% GC/MS confirmation rate for phencyclidine in their samples whenever radioimmunoassay (RIA) indicates the drug is present at or above 10 nanograms per milliliter. A different laboratory may only have a 30% confirmation rate in their samples at the same RIA value, using the same techniques. If so, the first laboratory is justified in setting a lower immunoassay detection threshold than the second.

In this example the difference between the laboratories could be due to a lower GC/MS limit of detection in the first, sample matrix differences, etc. Many factors other than the sensitivity of immunoassay itself can affect the relative performance of the two techniques.

Once set, the immunoassay detection threshold needs to be continually re-evaluated. It is important to monitor the confirmation rate of actual samples

responding at or near the immunoassay threshold. It is also important to note here that a 100% correlation between any immunoassay and GC/MS is unrealistic to expect. As mentioned, either technique may have problems with certain samples. Immunoassays are generally known to have some cross-reactivity and even the most sensitive mass spectrometer will fail to confirm a drug when co-extracted components prevent good chromatography. The detection threshold ought to be modified as laboratory conditions change. Improvements in the immunoassay specificity or lower limits of detection of the confirmatory test may justify the lowering of the immunoassay detection threshold. On the other hand, the reverse would likely necessitate raising the threshold. By continually monitoring the confirmation rate over time, a DUID laboratory is able to modify the detection threshold as needed.

APPLICATION OF DETECTION THRESHOLD PRINCIPLES IN VIRGINIA

Only blood samples are submitted for DUID testing in Virginia. RIAs, coated tube, and double antibody kits, are used to screen all DUID samples with alcohol concentrations at or below 0.10 grams/deciliter. There are six drug categories frequently encountered in our sample population that we have found to be appropriately addressed by RIA. Those are barbiturates, benzodiazepines, cannabinoids, cocaine, phencyclidine, and opiates.

An RIA calibration curve (spline) using multiple blood calibrators and controls is included with each series of unknowns. All are sampled and analyzed by RIA in duplicate. Any occasional lack of agreement between pairs necessitates duplicate retesting. We empirically define our detection thresholds as the RIA response above which more than half of the actual samples are confirmed positive by GC/MS. At least one calibrator and one or more controls at or near the defined RIA detection threshold are included with each series. Actual samples responding equal to or greater than our defined threshold are aliquoted (individually) for quantitative gas chromatography and GC/MS confirmation. Nitrogen phosphorus, electron capture, flame ionization, and, in some cases, mass spectrometry are used for quantitation. All positive results are confirmed at least qualitatively via electron impact GC/MS with multiple selected ions or total ion spectra. Any RIA response less than half of our defined detection threshold merits a "none detected" report for that drug class without further

testing. Any sample with an RIA response less than the threshold by up to 50% via spline calculation is processed in duplicate through the usual quantitation and GC/MS confirmation route. Both aliquots must yield consistent quantitative values followed by GC/MS confirmation before a positive report is issued.

We believe that this inclusion of selected "sub-threshold" samples for further examination and confirmation serves to minimize false negative results just as the duplicate confirmatory requirement for these samples safeguards against false positive reports. Any possibility of a random contamination during the quantitation/confirmation steps would be revealed by the duplication requirement. Also, this practice promotes frequent re-evaluation of the low side of the

immunoassay threshold. Over time as improvements in the technologies have lead to higher confirmation frequencies of subthreshold samples, the thresholds were lowered. Conversely if confirmation frequencies were observed to decline, RIA thresholds would be raised. Table 1 demonstrates our experience with RIA detection thresholds defined in this manner. Please note that the nominal drug concentrations as measured by RIA are often only loosely correlated with the actual measured values. Cross-reacting metabolites and other structurally related substances may elevate the RIA measurement. Table 2 indicates the range of concentrations as determined by the more specific techniques in addition to the observed frequencies of positive results. Demographic information provided on these drug positive cases by Virginia law enforcement agencies is presented in Table 3.

Table 1. GC/MS CONFIRMATION RATES OF DUID SAMPLES (BLOOD) SCREENED BY RIA					
Drug Class	RIA Detection	GC/MS Confirmation Rates			
	Threshold	<1/2	1/2 - 1	1 - 2	>2 x
Barbiturates (1)	500 ng/ml As Secobarbital	N.T.	10%	70%	90%
Benzodiazepines (2)	50 ng/ml As Oxazepam	N.T.	46%*	65%	91%
Cannabinoids (2) (THC/THCA)	40 ng/ml As THCA	N.T.	45%	76%	76%
Cocaine/B.E. (1)	300 ng/ml As Benzoyllecg.	N.T.	29%	78%	92%
Opiates (3)	40 ng/ml As Morphine	N.T.	29%	61%	82%
Phencyclidine (1)	10 ng/ml As PCP	N.T.	10%	82%	98%
NOTES: (1) Diagnostic Products Coat-A-Count (2) Diagnostic Products Double Antibody (3) Roche Abuscreen N.T. Not Tested * 2/3 of Confirmed, Low RIA-Positive Benzodiazepines Were Alprazolam					

**Table 2. DRUG CONCENTRATIONS IN VIRGINIA DUID CASES FROM
1988 THROUGH 1991**

Class	Drug	% Samples Reported Pos.	Blood Conc. Low (mg/L)	Blood Conc. Mean (mg/L)	Blood Conc. High (mg/L)	Blood Conc. Std. Dev. (mg/L)
PCP	PCP	16.7	0.003	0.044	0.42	0.032
Cannabinoids		26.4				
	THC	13.5	0.0004	0.003	0.023	0.0032
	THC Acid	24.5	0.0025	0.035	0.230	0.032
Cocaine		14.5				
	Cocaine	4.3	0.010	0.153	0.79	0.172
	Benzoyllecgonine	14.5	0.033	0.76	6.6	0.99
Benzodiazepines		13.8				
	Diazepam	9.8	0.017	0.461	2.5	0.433
	Nordiazepam	8.0	0.027	0.461	2.4	0.410
	Alprazolam	2.7	0.010	0.067	0.31	0.059
	Chlordiazepoxide	1.0	0.08	1.7	5.0	1.59
	Ndesalkyl Flurazepam	0.5	0.038	0.18	0.5	0.143
Barbiturates		7.1				
	Butalbital	4.9	0.60	8.4	27.0	6.20
	Phenobarbital	1.5	1.9	15	38.5	10.5
	Pentobarbital	0.6	0.8	3.19	14.1	3.63
	Butabarbital	0.2	0.04	5.32	10.4	4.39
Opiates		3.9				
	Codeine	3.5	0.01	0.19	1.35	0.26
	Morphine	2.2	0.001	0.036	0.37	0.06

**Table 3. 1990 AND 1991 DEMOGRAPHIC DATA ON VIRGINIA DUID
POSITIVE SUBMISSIONS**

Drug Class	Age Range (Yrs)	Median Age (Yrs)	Percent Males	Percent Caucasian
Barbiturates	20 - 60	34	51*	94
Benzodiazepines	16 - 60	32	70	94
Cannabinoids	16 - 60	27	92	88
Cocaine/Metabolite	16 - 51	29	78	67
Opiates	20 - 47	34	63	90
Phencyclidine	18 - 42	29	79	96
NOTE: *Only in butalbital positive cases did females exceed males by 53% to 47%.				

Application of Robotics and Automation to a Forensic Toxicology Laboratory

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Early in the spring of 1986, a campaign was begun to provide police officers within South Carolina an additional tool to combat the growing number of driving under the influence (DUI) cases. There was a common feeling among those involved that existing legislation failed to address the seriousness of DUI cases involving drugs alone or in combination with alcohol. Laws relating to DUI involving alcohol or drugs were by no means new to South Carolina, having had a statute in effect since 1949 (56 SC Code of Laws Sec. 5-2930. 1976). However, the existing statute provided no legal means by which an officer could request the collection of a urine or blood sample for the purpose of conducting a drug test. Over time, public opinion regarding the use of drugs, licit and illicit, had changed. Recreational use of illegal substances had risen to alarming levels while abuse of many prescription medications became commonplace as well.

On June 30, 1987, a bill was signed into law by Governor Carroll A. Campbell, Jr. (56 SC Code. Sec. 5-2950. 1987), which for the first time afforded officers the opportunity to request a sample of an individual's urine to be tested in cases of suspected drug use. The passage of this statute, commonly referred to as the Implied Consent Statute, addressed the officers' concerns regarding collection of samples, but failed to address the logistical question of how these samples would be processed. Over the 4 years preceding the passage of the Implied Consent Statute, the Toxicology Section of the South Carolina Law Enforcement Division (SLED) had experienced an average annual increase in total cases submitted of 12%, with little or no increase in personnel (see Table 1). To compound the existing personnel shortage and case backlog, it was estimated that passage of the Implied Consent Statute would result in an

**Table 1. TOTAL CASES RECEIVED
(1982 - 1986)**

Year	Total Cases Received	Increase
1982	1682	-----
1983	1903	13.1%
1984	1964	3.2%
1985	2289	16.5%
1986	2637	15.2%
Average Annual Increase - 12.0%		

additional 3,500 cases submitted annually to the Toxicology Section for analysis (SLED 1986).

The Forensic Services Administration, being fully aware of the difficulties involved in achieving full funding for a large increase in personnel, began a search for creative alternatives; alternatives which would allow a minimal staff to process a maximum number of samples within the shortest possible time. An ambitious program was begun in which SLED forensic services would become one of the first state-supported forensic laboratories in the country to attempt the implementation of laboratory robotics for the preparation (extraction) of biological samples for testing. Long-term goals included the purchase of automated data acquisition software capable of on-line or unattended compound identification, facilities for long-term data storage and retrieval in addition to a laboratory information management system capable of interfacing with existing equipment to provide on-line sample tracking and automated report generation (Wells 1991).

LABORATORY ROBOTICS AND ROUTINE SAMPLE PREPARATION

The very nature of a forensic laboratory requires rapid, accurate, and cost-effective methods of analysis in order to produce meaningful results in a timely fashion. When deciding which facet of laboratory activity to automate, careful attention was given to those techniques which were time-consuming, repetitious, or those which presented the greatest amount of potential for exposure to biological hazards. In each of these areas, laboratory robots have repeatedly proven their effectiveness.

Upon careful scrutiny of laboratory activity, it was commonly agreed that sample preparation or extraction, prior to analysis, presented the greatest challenge to providing timely results. In addition, by eliminating manual sample preparation, the hazards associated with exposure to biological specimens were greatly reduced. As such, it was decided that our initial envelope of activity would be centered on the development of a series of automated sample preparation or extraction protocols to be utilized with biological samples.

In the spring of 1988, requests for quotations and availability were sent to several capable vendors. Upon careful consideration of the proposals which were returned to our department, Zymark Corporation (Hopkinton, MA) was chosen as the supplier for the first pair of laboratory robots, associated peripherals, and software necessary for routine operation. Delivery of Zymark robots, which will be referred to for the purposes of this paper as SLED-1 and SLED-2, occurred in mid-July 1988.

INSTRUMENTATION

SLED-1 and SLED-2 consisted of identical Zymate II PyTechnology robots and associated hardware as indicated in Figure 1. Specific site requirements, supplied by our laboratory, included a source of compressed air for operation of pneumatic valves or pistons, a supply of high purity nitrogen for evaporation, and a regulated filtered power supply with battery backup. Special attention should be paid to item number 8, a ZP730-1 Liquid-Solid Extraction Pysection. At the time of our initial purchase, the liquid-solid extraction station was capable of utilizing only three solvents. Shortly thereafter, a six solvent liquid-solid

station became available. The original hardware and software have been upgraded to include this additional level of flexibility.

Upon installation of these systems, Claude Hatfield (Zymark Corporation, Hopkinton, MA) visited our facility to assist in the writing of the initial application software. The language utilized by Zymate II PyTechnology robots is known as Easylab, a proprietary language developed by Zymark. As the name implies, Easylab is designed to be user friendly and easily understood. However, with no prior experience nor training in the use of this language, writing the application software appeared an insurmountable task. The assistance provided by Mr. Hatfield was invaluable to me and our facility, substantially shortening the time required for development and implementation of this new equipment.

METHOD DEVELOPMENT

Two initial applications were selected for use in our laboratory. Application one, consisted of a liquid-solid extraction utilizing Prep Sep C18 columns (Fisher Scientific, Pittsburgh, PA), was provided by James L. Merdink, American BioTest Laboratories, Inc. (Santa Clara, CA). This application has proven to be exceptionally useful for cocaine and marijuana determinations from urine samples and is currently being utilized in the DUI section of our laboratory. Application two was to be a liquid-liquid extraction designed to recover acidic and neutral drugs from urine. With time, this application has been replaced by a second liquid-solid extraction which utilizes columns provided by Worldwide Monitoring Corporation (Horsham, PA).

These Worldwide columns, formally known as Clean Screen DAU, contain a proprietary packing which is well suited to the recovery of acidic, neutral, and basic drugs from biological samples in a single extraction. The methodology which is employed by the robot for this extraction (Table 2) was developed with assistance provided by the manufacturer and affords excellent recovery for the vast majority of commonly encountered drugs. With minor procedural changes, our laboratory is also able to utilize this method for the automated processing of whole blood, serum, plasma, and gastric samples within our DUI and Death Investigation Sections.

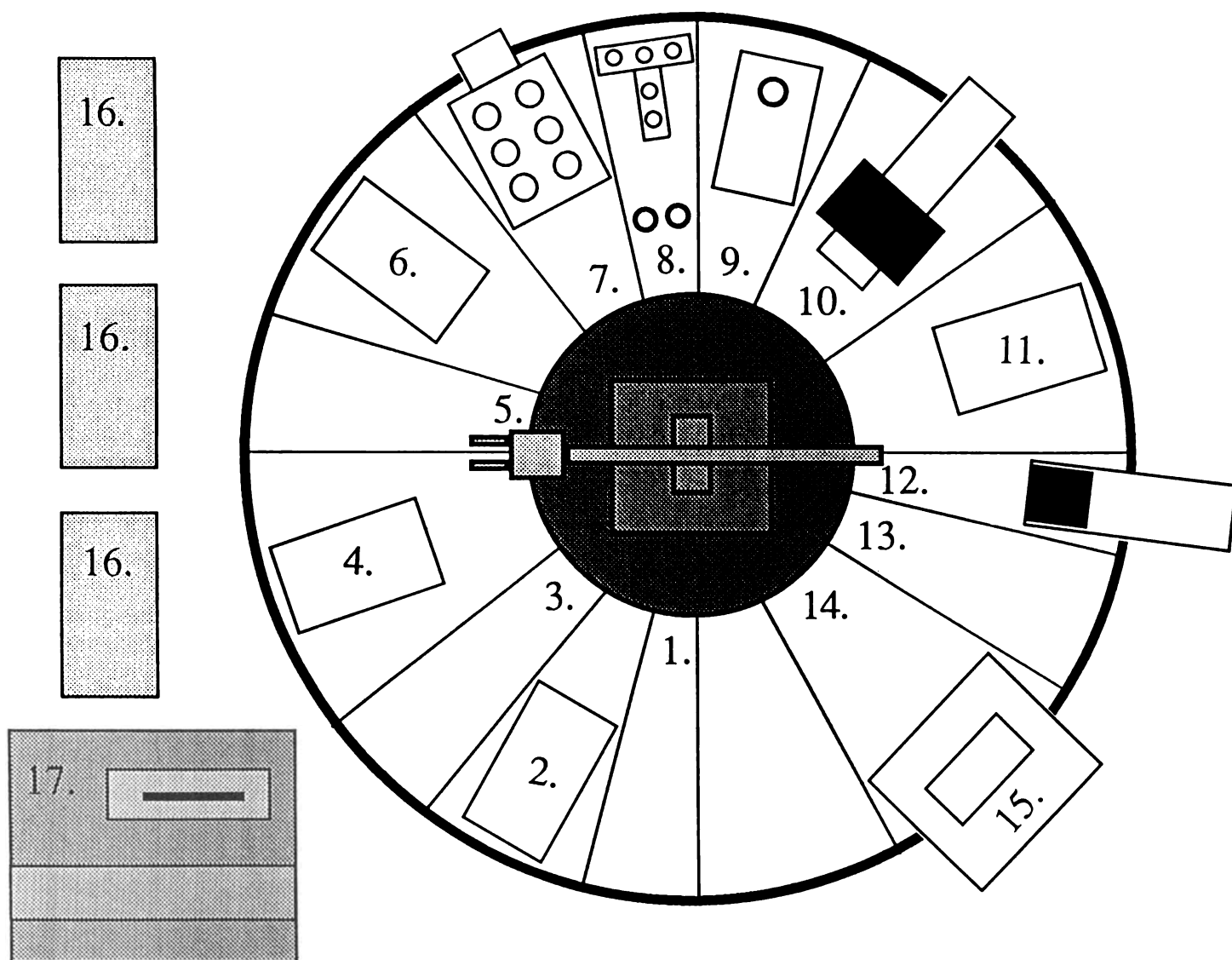


FIGURE I. Schematic of the Robot Configuration

- | | |
|------------------------------|--------------------------------|
| 1. GP Hand A | 10. 16 x 100 mm Tube Dispenser |
| 2. 16 x 100 mm Tube Rack | 11. Custom Incubation Station |
| 3. 0.2-2.0 mL Pipetting Hand | 12. Disposal Station |
| 4. 16 x 100 mm Tube Rack | 13. Liquid/Liquid Extraction |
| 5. Wisp Vial Filling Station | 14. Tumble Mixing Station |
| 6. Custom Covered Rack | 15. Centrifugation Station |
| 7. Evaporation Station | 16. Master Laboratory Station |
| 8. Liquid/Solid Extraction | 17. System V Controller |
| 9. Dilute and Dissolve | |

Items not depicted in above drawing:
2 Power and Event Controllers

Table 2. WORLDWIDE MONITORING DAU ROBOTIC EXTRACTION

1. SAMPLE PREPARATION

- To 5 ml of urine, add internal standard(s) and 2 ml of 1.0 M Sodium Acetate buffer (pH 6.0). (Sodium Acetate buffer contains B-Glucuronidase and should be prepared immediately prior to use.)
- Sample pH should be 6.0 ± 0.5 .
- Vortex sample.
- Incubate sample for 3 hours at 65°C.
- Centrifuge sample.

2. PREPARE CLEAN SCREEN DAU COLUMN

- 2 x 2.5 ml Hexane; air push.
- 2 x 2.0 ml MeOH; air push.
- 2 x 2.5 ml 0.1 M Phosphate buffer; air push.
- 1 x 1.0 ml 1.0 N Acetic Acid; air push.

3. APPLY SAMPLE

- Pour sample onto column and elute at rate not exceeding 1 - 2 ml/min.

4. WASH COLUMN

- 1 x 2.5 ml 0.1 M Phosphate buffer; air push.
- 1 x 1.5 ml 1.0 M Acetic Acid; air push.
- Dry column (5 min with maximum air push).
- 1 x 2.5 ml Hexane; air push.

5. ELUTE ACIDIC AND NEUTRAL DRUGS

- 2 x 2.5 ml Hexane/Ethyl Acetate (50/50). Collect fraction at 5 ml/min.

6. WASH COLUMN

- 2 x 2.5 ml MeOH; air push.

7. ELUTE BASIC DRUGS

- 1 x 2 ml MeOH/NH₄OH (98/2); collect eluate in a second, clean, 16 x 100 test tube at rates of 1 - 2 ml/min.

8. DRY ELUATE

- Dry acidic/neutral and basic fractions under a steady stream of Nitrogen. The evaporation temperatures should not exceed 34°C.

The final step(s) may involve a subsequent liquid-liquid extraction, sample derivatization or simple reconstruction in an acceptable solvent.

BENEFITS DERIVED FROM ROBOTICS

The inclusion of SLED-1 and SLED-2 into our work environment has produced a multitude of beneficial results, as well as some unexpected realizations. Since their inception, the robots have proven to be reliable sample manipulators, capable of unattended operation, thus allowing existing personnel an opportunity to participate in more challenging activities. By allowing existing laboratory personnel the time and opportunity to participate in method development, our robots have indirectly helped to create an atmosphere in which job satisfaction, creativity, and achievement abound.

It should be noted that laboratory robots are not the solution for all that ails modern forensic laboratories. The utilization of SLED-1 and SLED-2 has not resulted, as expected, in a reduction in the number of cases within our laboratory waiting to be processed. The use of laboratory robots, improved methods of extraction, and standardization of techniques have, instead, created an increase in the percentage of total cases which are confirmed positive for some drug or poison. Although specific statistics are unavailable at this time, it is conservatively estimated that 85% - 95% of those cases which screen positive via immunoassay are now routinely confirmed. This increased rate of confirmation has resulted in increased court time, thus causing the further reliance on laboratory automation.

AUTOMATED DATA ACQUISITION

The second phase of automation began with the purchase of several Hewlett Packard (HP Analytical Products Group, Palo Alto, CA) gas chromatograph/mass spectrometers (GC/MS) equipped with auto samplers. Each instrument was supplied with an after market software package, capable of automated data acquisition, sequenced acquisition, data reduction, and report generation known as TARGET Compound Analysis Software (Thru-Put Systems, Inc., Orlando, FL). Although originally designed for environmental testing laboratories, TARGET is well suited to the demands of modern toxicology laboratories. Some standard features provided by TARGET include password protection, to prevent unauthorized changes of operating parameters; user logs, which track an individual's activity while logged onto a system; and multiformat report generation, to meet the most critical demands for documentation. An example of TARGET's

versatility is demonstrated by the multitude of graphical reporting options available to the end user (see Figure 2).

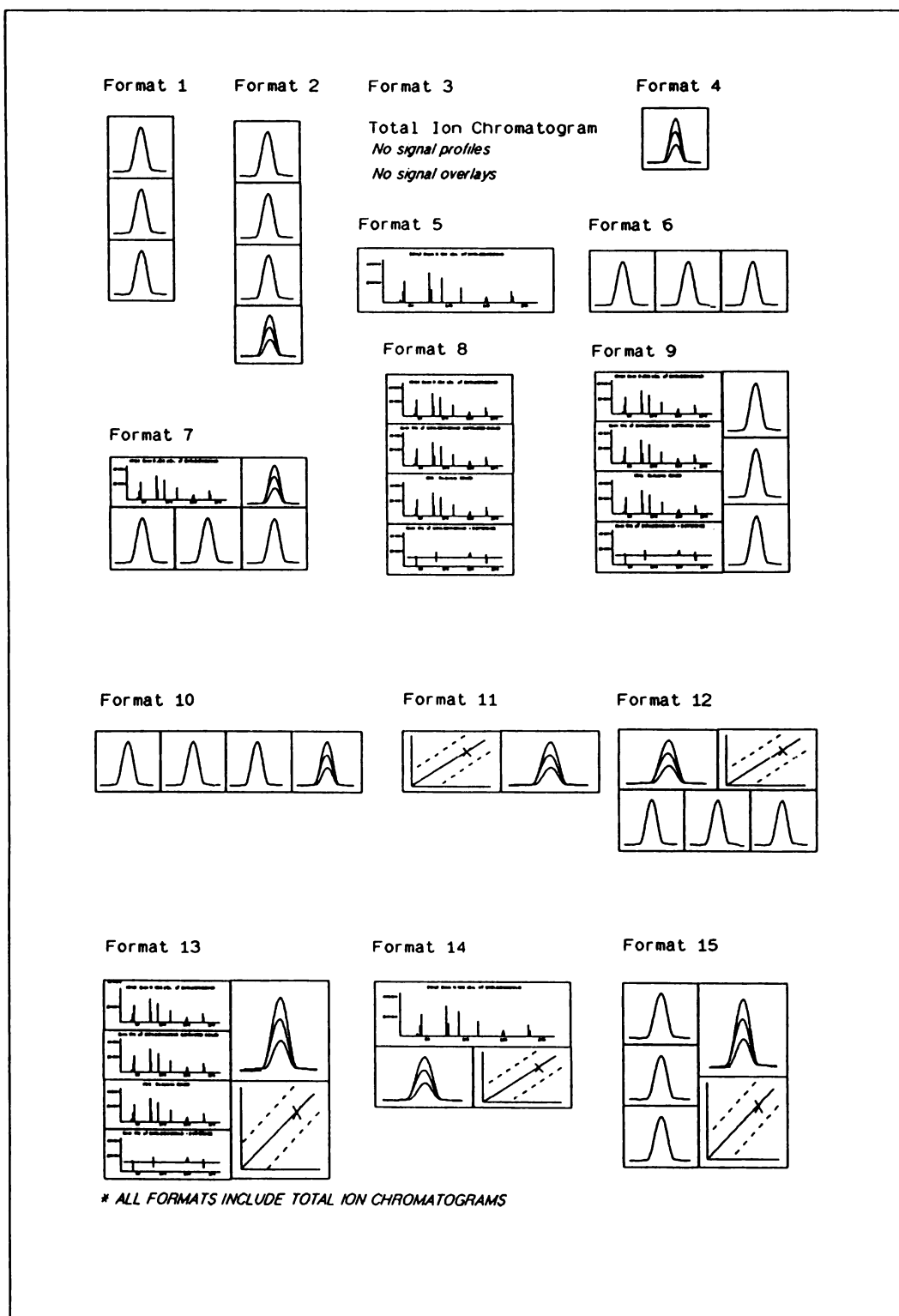
Running concurrently with TARGET is a second Thru-Put product known as Quality Base. Quality Base is capable of tracking instrument tune characteristics from multiple systems in an attempt to maximize the usefulness of preventive maintenance and minimize downtime. Generation of pertinent quality control data is facilitated through features supplied by Quality Base. Information available for long-term storage and retrieval includes various mass spectrometer operating parameters. Statistical treatment of these values may provide reports containing multiplier voltage or entrance lens voltage versus date. In addition, reports containing relative or absolute abundances of masses utilized in the tuning sequence versus multiplier voltage/date may be generated.

LONG-TERM DATA STORAGE AND RETRIEVAL

In an attempt to provide long-term data storage and retrieval capabilities, while keeping expenses to a minimum, the Toxicology and Drug Identification Departments have jointly implemented a local area network (LAN), linking instrumentation within these departments to two central HP Unix-based file servers. Within this LAN are located three HP 6300/650 optical drives which are utilized for the long-term storage of analytical data. Each optical disk has a capacity of approximately 300 MBytes per side and exists as if it were a removable hard drive. The specific linking of these drives is transparent to the user, causing only a minor reduction in access speed.

The Toxicology Department currently utilizes four HP 5988 GC/MS systems, and one HP 5970 MSD, each of which is controlled independently by an HP Pascal ChemStation. The file format of these stations is not compatible with that of the newer HP Unix-based stations; and, therefore, must undergo a conversion before transfer for long-term storage. HP ChemLan is the product which is responsible for this conversion and the subsequent transfer to optical disk. After each session of data acquisition, the previously defined method file invokes HP ChemLan to process the raw data from that acquisition and transfer the processed data file to one of the HP Unix ChemStations. Future review of this file may be accomplished through either the originating

FIGURE II. Target Graphic Reports



Pascal ChemStation or the HP Unix server located within the toxicology laboratory.

LABORATORY INFORMATION MANAGEMENT SYSTEM (LIMS)

The unresolved long-term goal involves the integration of a LIMS with existing LAN. As envisioned, this LIMS will be capable of providing immediate information as to the physical location and status of analysis for any given piece of evidence. Upon completion of required examinations and approval by the responsible toxicologist or chemist, a report will be generated automatically. Agencies which submit evidence to SLED Forensic Services will be allowed access via modem to ascertain the status or results of a given case. In addition, the incorporation of electronic mail will facilitate notification of upcoming court dates, allowing personnel to utilize laboratory time more efficiently.

CONCLUSION

The process of automation or modernization of the Toxicology Department has, in fact, had an impact on nearly every department within SLED Forensic Services. In the 3 years following the purchase of SLED-1 and SLED-2, the Toxicology and Drug Identification Departments have acquired 3 additional Zymark PyTechnology laboratory robots. All five systems are currently utilized for sample preparation prior to GC/MS confirmation. The process continues as new products become available which are capable of providing enhanced sample tracking or productivity. One example involves the addition of a bar code reader to the robotic systems. The bar code reader is capable of scanning 16 x 100 mm test tubes and 11 mm GC vials to provide a complete sample history, which includes the initial and final bar code number. In the near future, the bar code numbers from the final GC vial will be sent to the GC/MS sample table via LAN, thus reducing time spent manually entering this information.

As is indicated throughout this paper, the introduction of automation to our laboratory is a process which has spanned almost 4 years and is not yet complete. The ambitious goals set forth by the SLED Forensic Services Administration are not unlike those of other laboratories across the country. Experience gained throughout this process has introduced us to

several concepts which we feel would assist others interested in the implementation of this type of program:

1. Before attempting to automate any procedure, a valid manual method should exist.
2. Successful implementation of robotics/automation requires the acceptance and participation of all laboratory personnel. It is, therefore, essential that personnel be kept informed of upcoming deadlines and be allowed to participate during system installation and validation.
3. Before attempting a program of this nature and magnitude, a list of realistic goals and deadlines should be recorded for future reference.

Most of all, it is important to understand that robots or other forms of automation within the laboratory cannot replace qualified personnel. At best, the various forms of automation merely function to improve productivity. In performing tasks which were previously carried out by the individual, robots allow existing personnel added time to participate in activities which are important to the continued growth of the individual and the laboratory as a unit. Robotics and other forms of laboratory automation should be viewed in the context for which they were designed—as tools capable of assisting laboratory personnel in their daily activities.

ACKNOWLEDGMENTS

The implementation of automated sample preparation and analysis within our laboratory is today a reality. Currently, routine extractions are performed with the aid of robotics, thus providing additional time for the individual to undertake more enjoyable and challenging tasks. This project could not have been undertaken without the support and cooperation of many individuals: Claude Hatfield, Carl Scharicz, and Justin Webster from Zymark; Julie Tomlinson from Glaxo; Gerald Long and Ed Heabner from Worldwide Monitoring; Lynn Mathews from Thru-Put Systems; and James Merdink from American Biotest Laboratories. In addition, I would like to thank those individuals whose daily assistance have made this project a success, including the administration of the SLED, members of the Forensic Services Administration, and the Toxicologists who operate these instruments on a daily

basis. Also, I would like to offer a warm word of thanks to Debbie Banks for her invaluable assistance during the preparation of this manuscript, Lt. Roy Paschal for his assistance in preparing the figures which bring meaning to the descriptions contained within the body of this text, and Lt. Ken Habben whose understanding and guidance throughout the implementation of laboratory automation have made my task much more enjoyable.

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Overview and History of Workplace Drug Testing

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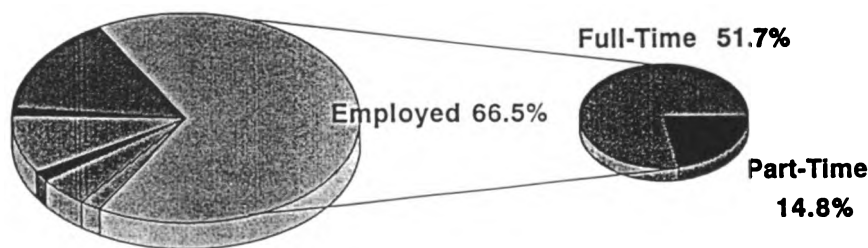
Drug use and its corresponding impact on the workplace continue to be a serious and elusive problem in America. Tragic events periodically provide notorious examples of how a single such incident may significantly impact society as well as render long-term economic and environmental consequences. Dramatic examples such as the 1986 railroad accident in Chase, Maryland, have focused the public's attention on how drug and alcohol use can impact the lives of many others, and ultimately, the health of our society (Gust *et al.* 1991).

The public response to such tragic and preventable events has continued to aid the campaign to reduce drug abuse. The portion of this campaign which appears to have had the most significant impact in the reduction of illicit drug use is the effort to achieve drug-free

workplaces (Gust *et al.* 1991). Data from the National Institute on Drug Abuse's (NIDA's) 1991 National Household Survey on Drug Abuse indicates that a majority (approximately 66.5%) of illicit drug users over the age of 18 are employed; while only 15.2% are unemployed (Figure 1). The remaining 18.3% are categorized as homemakers, students, disabled, retired, and other. Of the 66.5% that are employed, 51.7% are employed full-time and 14.8% are employed part-time (Figure 2) (NIDA 1992).

The federal government and a large number of private sector businesses have responded by establishing programs focused on the prevention of drug use and the treatment of drug users.

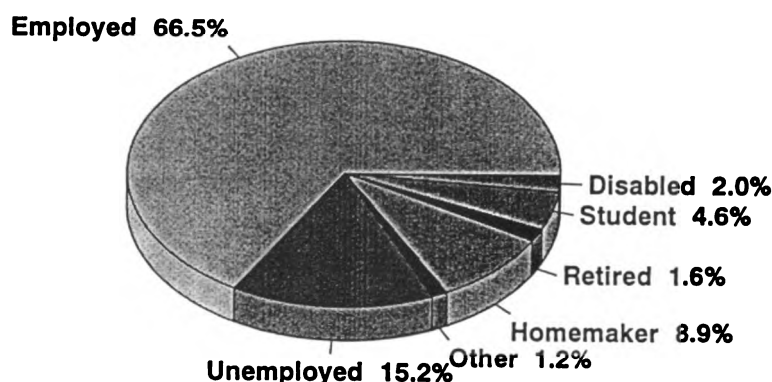
The Majority of Current Drug Users Are Employed (Current Users of Illicit Drugs, Ages 18 and Over)



Source: National Institute on Drug Abuse
National Household Survey on Drug Abuse, 1991
Prepared by: Division of Applied Research, NIDA

Figure 1.

The Majority of Current Drug Users Are Employed (Current Users of Illicit Drugs, Ages 18 and Over)



Source: National Institute on Drug Abuse
National Household Survey on Drug Abuse, 1991
Prepared by: Division of Applied Research, NIDA

Figure 2.

FEDERAL INITIATIVES

In 1986 Executive Order 12564 (President, Executive Order 1988) directed each federal agency to develop a comprehensive program to achieve a drug-free workplace for federal employees. The President also presented it as a model for the private sector. This is a comprehensive model requiring that the prohibition against illegal drug use and its consequences be spelled out and effectively communicated to employees: that employees be educated about the dangers of drug use; that supervisors be trained concerning their responsibilities; that a helping hand in the form of an Employee Assistance Program (EAP) be available for employees who have a drug problem; and finally, that there be provisions for identifying illegal drug users, including drug testing on a controlled and carefully monitored basis.

It is unequivocal in stating that federal employees must refrain from the use of illegal drugs; that their use, whether on or off duty, is contrary to the efficiency of the service; and that persons who use illegal drugs are not suitable for federal employment. An agency may decide to provide a "safe harbor" [*i.e.*, take no action against any employee who: (1) voluntarily identifies herself/himself as a user; (2) obtains counseling or rehabilitation; and (3) thereafter refrains from using

illegal drugs]. However, in the absence of this set of conditions, agencies must initiate action to discipline any employee who is found to use illegal drugs. Further, agencies must initiate action to terminate any employee who uses illegal drugs and who refuses to get help or stop using.

In 1987 Public Law 100-71 placed certain conditions on drug testing of federal employees by requiring the Secretary of Health and Human Services (HHS) to set comprehensive standards for all aspects of laboratory drug testing and laboratory procedures and requiring the use of the best available technology for ensuring the full reliability and accuracy of drug tests and strict procedures governing the chain of custody of specimens collected for drug testing. The standards must state the drugs for which federal workers can be tested and set scientific and technical standards for drug testing and for the certification of laboratories which conduct drug tests of federal workers.

In accordance with Public Law 100-71 the Secretary issued the mandatory guidelines for Federal Workplace Drug Testing Programs which set scientific and technical standards for drug testing of federal employees and for certification of drug testing laboratories. The "gold standard" set by these guidelines has required good laboratory practices resulting in

accurate and reliable drug testing. Employers in both public and private sectors are insisting on those high standards because of their reliability and because they provide a formidable defense against legal challenges based on the accuracy of testing.

The mandatory guidelines are composed of three subparts. Subpart A states who is covered by the guidelines, definitions of terms in the guidelines, and how revisions will be handled. Subpart B of the guidelines calls for applicant and random testing of five drugs: marijuana, cocaine, opiates, amphetamines, and phencyclidine. Federal agency applicant and random drug-testing programs must at a minimum test for marijuana and cocaine. They are also authorized to test applicants and persons in testing designated positions for opiates, amphetamines, and phencyclidine.

The guidelines require that there be a designated collection site and that it be secure, permitting entry only of authorized personnel. Each individual is afforded privacy for the collection unless there is a specific reason to believe that an individual may alter or substitute the specimen. [For example, if a specimen falls outside the prescribed temperature range there is "reason to believe" that the individual may have altered or substituted the specimen and another specimen must be collected as soon as possible under direct observation of a same gender collection-site person. (A higher-level supervisor must approve "direct observation.")]. Both specimens are tested.

The guidelines require strict chain of custody (*i.e.*, documentation to account for the identity and integrity of each urine specimen from point of collection to final disposition). At the collection site, identifying seals and labels are attached to the specimen bottle in the presence of the collection-site person and the individual being tested. The individual then initials the labeled bottle to acknowledge that it is the specimen collected from him or her. Each time custody is transferred, the chain of custody form must show the date and purpose of the transfer and the initials or name of the individual receiving the specimen to maintain accountability for the specimen.

The guidelines require three separate and distinct Quality Assurance/Quality Control (QA/QC) measures:

1. The laboratory must provide its own internal controls. The guidelines require that each

analytical run include specimens certified to contain no drug; specimens fortified with known standards; and positive controls at or near the cutoff. A minimum of 10% of all samples must be QA/QC specimens. They should appear as normal samples to laboratory analysts.

2. Federal agencies submit blind performance test specimens to the laboratories with which they contract. After the initial 90-day period of any drug-testing program, the blind performance test specimens submitted by the agency must equal at least 10% of all samples submitted (up to a maximum of 250) per quarter. Eighty percent must be blank (*i.e.*, contain no drug) and the remaining must be spiked with one or more drugs distributed such that all drugs to be tested are included in roughly equal numbers. In response to unsatisfactory laboratory performance, taking the nature of the error into consideration (*i.e.*, whether administrative, methodological, or a less serious error which has been corrected), the Secretary has the option of revoking or suspending the laboratory's certification or recommending that no further action be taken.
3. NIDA's National Laboratory Certification Program requires performance/proficiency testing in order to become a certified laboratory and continues that proficiency testing for the life of the certification. To be initially certified, an applicant laboratory must successfully complete three cycles of testing (*i.e.*, correctly identifying and confirming 90% of the total drug challenges for each shipment submitted by the NIDA certification program). A false positive will immediately disqualify an applicant. Further, applicant laboratories must successfully quantitate the five NIDA drugs in accordance with the guidelines on at least 50% of the challenges. Quantitative values that differ by more than 50% will result in disqualification. Proficiency testing continues after certification on a bimonthly basis. Failure to maintain a grade of 90% on any required test cycle may result in suspension or revocation of certification. No false positives are permitted for certified laboratories and may result in suspension or revocation of certification.

Also in connection with QA/QC requirements, it is significant that prior to certification a team of inspectors conducts an on-site inspection of laboratories in the National Laboratory Certification Program.

The mandatory guidelines are explicit in making the Medical Review Officer (MRO) the gatekeeper in maintaining the integrity of the standards set for the federal drug-testing program. The MRO's leading role was created in recognition of the fact that a positive result reported out of the laboratory does not automatically identify an individual as an illegal drug user. The MRO is charged with reviewing and interpreting positive test results. He/she must afford the individual an opportunity to discuss a positive result reported by the laboratory. He/she must take into consideration all medical records made available by the tested individual when a laboratory positive could have resulted from legally prescribed medications. The physician/MRO inserts his or her knowledge of possible alternative medical explanations and best medical judgement in deciding whether to report a laboratory positive back to the agency as a positive or negative—and he or she does so only after reviewing and verifying the laboratory results.

Also in 1988, the Department of Defense issued regulations (48 CFR Parts 223 and 252) (DOD 1988) which require defense contractors to “institute and maintain a program for achieving the objective of a drug-free work force.” It is aimed at ensuring national security through drug-free work environments; for example, those workplaces that affect the safety and reliability of weapons systems. The regulations apply to all contracts which require access to classified information or where the contracting officer determines it necessary for reasons of national security or to protect the health or safety of those who perform the contract or of those who use or are affected by the product of the contract. The Department of Defense enforces drug-free workplace requirements imposed on its contractors through normal remedies for noncompliance, including terminating the contract or suspending or debarring the contractor.

In 1988 the Department of Transportation (DOT) published regulations (49 CFR Part 40) (DOT 1989) which impact approximately four million employees regulated by the six separate agencies within DOT: the Federal Railroad Administration, the Federal Highway Administration, the Federal Aviation Administration, the Research and Special Programs Administration

(Natural Gas, Liquefied Natural Gas, and Hazardous Liquid Pipeline Operations), the Coast Guard, and the Urban Mass Transportation Administration. The Urban Mass Transportation Administration's regulation has been withdrawn pending approval of statutory authority for the regulation. Each of these agencies has issued separate rules applicable to private sector employers under their jurisdiction. While there are substantive and editorial differences in each agency's rule, they have the following provisions in common:

- Require employee education and supervisor training;
- Require availability of EAPs;
- Require that a MRO be designated;
- Permit employer discretion with regard to rehabilitation and discipline;
- Require preemployment testing for safety-sensitive positions;
- Authorize testing for the same five drugs as the mandatory guidelines;
- Require testing of persons in safety-sensitive positions on a random basis, as part of required periodic physicals, for reasonable cause, and after a serious accident or incident.
- With some industry specific modifications, the mandatory guidelines apply through DOT with the result that only NIDA-certified laboratories may be used.

In 1988 the Drug-Free Workplace Act (DFWA 1988) conditioned receipt of all federal grants and contracts to individuals and all contracts for over \$25,000 on the recipient's certification to provide a drug-free workplace. Its reach is broad (*i.e.*, it is not limited to sensitive employees, but it is not as deep as other federal initiatives): in particular, it does not require drug testing. It applies only to prime contractors and direct grantees and is implemented through the granting and contracting rules and procedures of each agency subject to guidelines issued in May of 1990 by the Office of Management and Budget (OMB 1990). Under the act, grantees and contractors must take the following steps to achieve a drug-free workplace:

- Publish a statement, a copy of which must be given to each employee, notifying him/her that the unlawful manufacture, distribution, dispensation, possession, or use of a controlled substance is prohibited in the workplace and stating the actions that will be taken if that prohibition is violated.
- Establish a drug-free awareness program of the dangers of drug abuse, the grantee/contractor(s) policy on drug abuse, availability of an EAP, rehabilitation, and counseling services, and penalties for violation of the published prohibitions.
- Specifically notify all employees in writing that as a condition of employment they must abide by the terms of the employer's published statement and notify the contractor of any conviction under a criminal drug statute for a violation occurring in the workplace no later than 5 days after the conviction. The employer must notify the relevant federal agency within 10 days after receiving notice.
- The employer has 30 days after notification to take appropriate action, up to and including termination or to require the employee to participate in an EAP or rehabilitation program.
- The employer must continue good faith efforts "to maintain a drug-free workplace."

The penalty for making false certifications, for violation of certifications, or failure to make good faith efforts to maintain a drug-free workplace are substantially the same for grants and contracts and may include suspension of payments, termination for default, and suspension or debarment for up to 5 years.

In 1989 the Nuclear Regulatory Commission (NRC) published regulations (10 CFR Parts 2 and 26) affecting licensees of the NRC authorized to operate nuclear power reactors (NRC 1989). Licensees are required by these regulations to implement fitness-for-duty programs. While it addresses other aspects of fitness, it is significantly aimed at creating a drug-free work environment within nuclear power plants. It is similar in most aspects to programs implemented by the DOT but adopts provisions of the mandatory guidelines, with an awareness "that the guidelines, as

written by HHS to apply to testing by federal agencies, do not perfectly fit the circumstances of the licensees regulated by the NRC." NRC permits tests for initial drugs, permits lowering of cutoffs for initial screening tests, and permits limited employer access to the results of initial screening tests.

In the fall of 1989 NIDA held a Technical and Scientific Review of the mandatory guidelines. We have recently proposed changes to the guidelines based on NIDA's first 4 years of experience in implementing and administering these guidelines and the experiences of those regulatory agencies citing these guidelines in their rules.

The proposed changes are as follows:

- Specimen volume will be reduced from 60 ml to 30 ml.
- Optional procedure for collecting "split specimens."
- Allowing federal agencies to use an individual, other than a collection-site employee, to observe the collection of a specimen whenever there is reason to believe the individual may have altered or substituted the specimen.
- Reducing the initial test level for marijuana metabolites from 100 ng/ml to 50 ng/ml.
- Permitting a laboratory to use multiple immunoassay tests for the same drug or drug class.
- Establishing the requirement that, in order for a specimen to be reported positive for only methamphetamine, it must also contain the metabolite amphetamine at a concentration equal to or greater than 200 ng/mL by the confirmatory test.
- Reducing the number of blind samples a federal agency must submit each quarter to its contracting laboratory from 10% of all samples to a minimum of 3%.
- Reducing the frequency of PT challenges from six cycles per year to four cycles per year.

- Establishing restrictions on the types of arrangements that can exist between the MRO and the laboratory.
- Adding a new Subpart D to the guidelines that would establish detailed procedures for the review of a suspension or proposed revocation of a certified laboratory.
- Providing that written notice of the suspension which is sent to the laboratory will be made available to the public upon request, as well as the reviewing official's written decision upholding or denying suspension or proposed revocation under the review procedures in Subpart D.
- Permitting a laboratory to use a certifying scientist who is only certified to review initial drug tests which are negative.

To date, President Bush has presented four National Drug Control Strategies to the Congress and to the American people. The first of these was in September 1989, the second in February 1990, the third in February 1991, and the most recent in January 1992. Taken together, these strategies acknowledge drug abuse as a significant public policy issue associated with rising rates of violent crime, serious damage to the nation's health and economy, and a key tension in international relationships. They propose an integrated series of federal initiatives to reduce the supply of drugs available in the United States and to reduce the demand for drugs by drug users.

The fourth National Drug Control Strategy reaffirms the elements of the first three strategies: "The overall objective of the strategy is to reduce drug use. This is to be accomplished by reducing both the supply of and demand for drugs ... We will keep the pressure on to prevent Americans, especially our children and young adults, from ever starting to use drugs. We must also expand our efforts with adults in the workplace and in the populations that are most resistant to the antidrug message." Unlike the previous strategies, the fourth strategy includes provisions to specifically sharpen "the focus on the treatment and prevention of alcohol abuse" and to prevent a resurgence of heroin use as the "availability and purity of heroin increase" (White House 1992).

Reducing drugs in the workplace is a key component of the third and fourth strategies. Every employer, large or small, is encouraged to establish a comprehensive workplace drug prevention program. New federal initiatives are anticipated to focus on helping small businesses (<100 employees) which comprise nearly 97% of the businesses in the United States, develop drug-free workplace programs. The development of model programs for small businesses is expected to focus on developing ways to make drug-free workplace programs affordable and cost-effective for such small businesses. For example, one approach has been the development of a consortium of small businesses for the purchase of drug-testing services, EAP services, and MRO services. These efforts highlight the administration's conviction that illicit drug abuse is a national problem affecting all strata of American society.

As previously mentioned, Executive Order 12564 directed each agency to develop a comprehensive program to achieve a drug-free workplace for federal employees. What components make up a comprehensive program?

- A clearly articulated written policy describing the employer's expectations about drug use and consequences of policy violations.
- An EAP to provide confidential problem assessment, counseling, referral to treatment, support, and guidance through the treatment/rehabilitation period and follow-up support after treatment.
- Supervisor training to orient supervisors to the agency's drug-abuse policy, to define the supervisor's responsibility to refer employees when job performance deficits are noted, to provide information on the workforce, to recognize and respond to employees with problems, to provide guidance on how to document changes in employee performance or conduct, and to explain the roles of the EAP and drug-testing components.
- Employee education to describe the signs and symptoms of drug abuse and effects on performance and to explain the program, including use of the EAP.

- Provisions to identify illegal drug abusers including drug testing on a controlled and carefully monitored basis. Six types of testing are included in the federal program: applicant, accident or unsafe practice, reasonable suspicion, follow-up to treatment, random, and voluntary.

RELATIONSHIP OF DRUG USE TO PERFORMANCE AND PRODUCTIVITY

Drug use has been shown to significantly impact worker performance, productivity, and safety. This is evidenced by an increase in job turnover rates, absenteeism, accidents and injuries, health care utilization, and theft/security problems. Lost productivity and poor employee morale may also result from employee drug use.

Recent studies indicate that performance and productivity are significantly influenced by illicit drug use. In June 1991 the U.S. Postal Service (USPS) conducted a follow-up study of employees testing positive for drugs at the time of preemployment urine drug testing (3.3 years earlier) versus those employees testing negative. Results of this study found that absenteeism rates were 66% higher (11.39% v. 6.85%) and employment termination rates were 77% higher (23.64% v. 13.35%).

The USPS study also determined that “when the involuntary turnover and absenteeism savings are combined, the estimated cost savings from screening out drug-positive applicants is nearly \$105 million for one cohort of employees ...” (N=180,000) over their employment tenure (Normand *et al.* 1990).

A similar study conducted by the Georgia Power Company of all workers (versus new hires in the USPS study) found that the absenteeism and medical benefits usage rates for employees testing positive were also significantly higher (Sheridan and Winkler 1989).

RECENT AND PENDING LEGISLATION

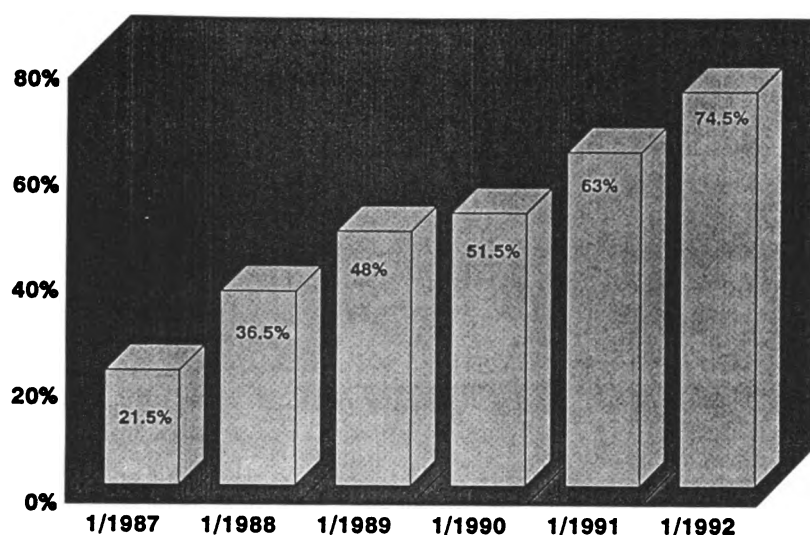
Several pieces of legislation which deal with a drug-free workplace or some component of it have been proposed (House Resolution 33 and Senate Bill 2008) and, in the case of the Americans with Disability Act and the Omnibus Transportation Employee Testing Act, ratified.

In 1990 during the 101st Congress, Representatives Thomas J. Bliley and John D. Dingell introduced House Resolution Number 33 (Drug Testing Quality Act 1991) which provided for certification of laboratories for the private sector using standards which are the same as those used for federal employees. HR 33 was originally passed as an amendment to the Crime Bill in the 101st Congress but was withdrawn prior to final passage and was reintroduced in the 102nd Congress with some modifications. The bill places emphasis on fitness/impairment for duty—regardless of whether a person is taking an illicit or a licit drug.

On November 21, 1991, Senators Hatch and Boren introduced Senate Bill 2008 (Quality Assurance in the Private Sector Drug Testing 1991) which calls for the establishment of federal standards to ensure quality assurance in private sector drug-testing programs to ensure uniformity and consistency in the application of such federal standards to preempt state and local laws and such other requirements that regulate private sector workplace drug-testing programs.

The Americans with Disabilities Act (ADA 1990), which prohibits discrimination against individuals with disabilities in employment, public services and transportation, public accommodations, and telecommunications services, also impacts on drug-free workplace efforts. In July 1992 the ADA will cover employers with 25 or more employees. In July of 1994 employers of 15 or more workers will be covered. Covered employers may not discriminate against a “qualified individual with a disability” in regard to job applications; hiring; advancement; discharge; compensation; training; or other terms, conditions, or privileges of employment. Employers may not refuse to hire an applicant because of a disability. Furthermore, employers may not fire a worker who develops a disability, including a disease such as cancer or AIDS, so long as that individual remains qualified and able to perform the duties of the job with reasonable accommodations. An individual with a disability is one who “has a physical or mental impairment that substantially limits one or more major life activities (e.g., caring for oneself, walking, seeing, hearing, speaking, working, a record of such impairment or who is regarded as having such an impairment).” The qualified individual with a disability is one who with or without reasonable accommodation, can perform “essential functions” of the job.

Workplace Drug Testing, 1987 - 1992

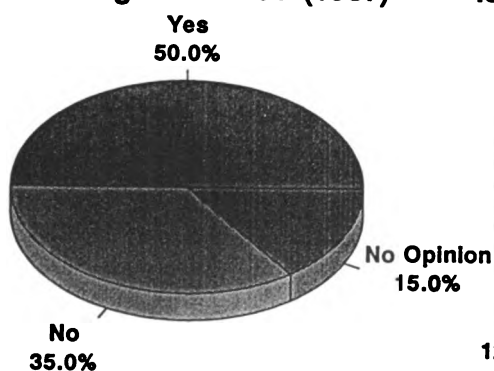


Source: American Management Association
"Workplace Drug Testing and Drug Abuse Policies," 1992.

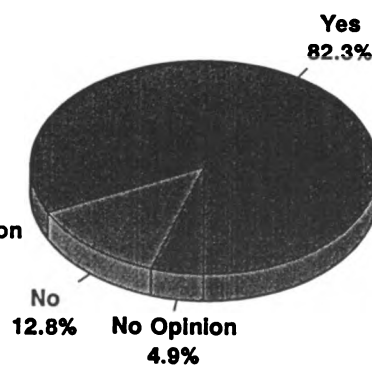
NIDA

Figure 3.

Is Testing Effective? (1987)



Is Testing Effective? (1992)



Source: American Management Association,
"Workplace Drug Testing and Drug Abuse Policies," 1992.

NIDA

Figure 4.

Employers can prohibit the use of alcohol and illegal drugs at the workplace, can require that employees not be under the influence of alcohol or illegal drugs on the job, and can discharge any employee for violations of these requirements. The ADA does not impact an employer's ability to perform drug testing for applicants or employees. Drug tests are not medical examinations under the ADA.

On October 28, 1991, President Bush signed HR 2942, the "Department of Transportation and Related Agencies Appropriations Act, 1992." Title V of the act, entitled the "Omnibus Transportation Employee Testing Act of 1991" (OTETA 1991) requires drug and alcohol testing of safety-sensitive employees in the aviation, rail, truck, bus, and mass transit sectors. With regard to drug testing, the act requires the transportation industries to conduct preemployment, reasonable suspicion, random, and postaccident testing; and all drug testing is to adhere to the provisions of the mandatory guidelines and any subsequent amendments.

The act also calls for the elimination of the use of alcohol and illicit drugs on or off duty by individuals involved in the operation of aircraft, trains, trucks, and buses; and states that rehabilitation efforts are critical to the testing program's success. As you may know, the impact of alcohol use in the workplace parallels that of drugs in the workplace. Data from NIDA's 1991 National Household Survey reveals that over 103.2 million people age 12 and over are current (past month) drinkers of alcohol, and 138 million people age 12 and over drank alcohol in the past year (NIDA 1992). With regard to alcohol testing, the act does not specify what method of testing should be employed (*i.e.*, breath, blood, combination of the two). It does require that the Secretary of Transportation develop regulations in consultation with the Secretary of HHS to "ensure appropriate safeguards for testing ... breath and body fluid samples, including urine and blood." The conference committee's report encouraged DOT to conduct alcohol testing on a performance-related or fitness-for-duty basis.

DRUG TESTING — THE PROGRESS

Recent data released by the American Management Association (AMA) indicates that workplace drug testing appears to have proven its worth to most employers (Figure 3). Certainly the increase in the percentage of practitioners who consider testing to be an effective way

of combating workplace drug abuse bears testimony to this observation. Moreover, the survey indicated that 48% of those companies that do not drug test express approval of drug testing (AMA 1992).

Over the past 5 years, the AMA estimates that workplace drug testing has increased a dramatic 250% from a modest 21.5% in 1987 to the most recent figure of 74.5% in 1992 (Figure 4). The greatest change, however, may be in the policies that determine who is tested and for what reasons. In 1987 the majority of companies that drug tested did so only when performance indicated drug abuse. The number of companies that have incorporated random, periodic, and/or preemployment drug testing has increased tenfold since 1987 (2.5% in 1987 v. 27.8% in 1992) (AMA 1992).

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Determination of Toxic Metals in Human Tissue

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Toxic metals are usually defined as those elements which disrupt metabolic processes. In this talk, I will use the term toxic metal more generally to refer to elements of concern in criminal poisoning cases, although not all of them are metals. Criminal poisoning cases involving toxic metals occur infrequently and the symptoms of toxic metal poisoning often mimic other common conditions, such as stomach flu. As a result, symptoms of toxic metal poisoning may not initially be recognized by clinical personnel and less than optimum tissue samples collected or after-the-fact determinations may be requested of the forensic laboratory. When a specific toxic metal is suspected and appropriate specimens are obtained, analysis is usually straightforward. For example, clinical laboratories frequently assess environmental or industrial exposure to metals such as lead by blood analysis. Reliable analytical procedures are well established for this type of determination. This paper discusses two aspects of toxic metal analysis which we have encountered in the FBI Laboratory, but for which well-accepted methods are not widely available. First is the question of selecting an analytical method for screening tissue specimens for the presence of high amounts of toxic metals when the specific toxin is not previously known. Second is the determination of the duration of exposure based on the axial distribution of toxic metals, notably arsenic, in hair.

TOXIC METAL SCREENS

A difficult problem in toxicology is the detection of toxic metal poisoning when there is limited clinical indication of which particular toxic metal, if any, is involved. In some instances, the decision to look for the presence of elevated levels of toxic metals is made after considerable time has elapsed following exposure. Those responsible for interpreting the significance of the analytical results must be concerned with collection

of the appropriate sample; prevention of contamination during sample collection, packaging, and transport; and obtaining correct literature data on which to base an opinion. The major concern of the analyst is providing accurate results for the nearly 30 possible toxic elements at the concentrations relevant to the tissues provided. To obtain accurate results, the analyst must use proper calibration procedures, minimize interelement or matrix effects, and follow appropriate quality assurance protocols. Excellent discussions of the pitfalls of analysis of tissue samples are given by Iyengar (1991) and Cornelis (1991). These two articles should be required reading for anyone attempting biokinetic studies of toxic metals.

There are several important considerations which must be taken into account in developing an effective screening method for detection of toxic metal poisoning. These include:

(1) **Choosing Appropriate Samples.** It is not possible to collect a single tissue sample which is appropriate for every toxic metal, since target organs vary from one element to another, for different forms of the same element, for different ingestion routes, and as a function of time after exposure. While metabolic pathways are well known for some toxic metals, this is not always the case, particularly when the mode of intake or form of the toxic metal is not known. Reference texts, such as Clarkson *et al.* (1988) give good discussions of several documented pathways and are helpful in selection of appropriate tissues for analysis. The critical organ from the standpoint of human health effects may not be the same as the optimum organ for detecting the occurrence of poisoning. Some metals cause shutdown of metabolic pathways at very low target tissue levels, yet may be concentrated at much higher levels in tissues of excretory organs, such as the kidneys. For example, bismuth, iron, and silver can be highly concentrated in

the kidneys without renal impairment, while cadmium, lead, mercury, and vanadium produce nephrotoxic effects with chronic exposure at relatively low levels.

In cases of acute poisoning by oral ingestion when samples can be taken quickly, the best samples generally are stomach and intestinal contents, blood, and urine. For chronic exposure or when relatively long periods of time have passed following exposure and toxic metals have had a chance to accumulate in the victim's tissues, the kidney or liver are the organs of choice for screening purposes as they concentrate most toxic metals. Specific examples of alternative tissues are bone for thallium, hair for arsenic, and ocular fluid for barium. In most instances of chronic or lethal exposure, toxic metal concentrations in the kidney are abnormally high and readily detectable. When toxic metal poisoning is suspected, but specific metals or the mode of administration are unknown, a variety of tissue samples including blood, urine, stomach contents, kidney, liver, lungs, and brain should be collected. In some instances of long-term, low-level exposure, bone, hair, and nails are the tissues of choice. When the victim is living, the choice of tissues which can be obtained is more limited. For exhumed bodies, tissue selection is generally limited to hair, bone, skin, and, if available, body cavity fluid.

(2) Avoiding Contamination or Compromise of Sample Integrity During Sample Collection, Transport, and Analysis. Modern analytical techniques have become so sensitive that contamination is noted now that would not have been detectable several years ago. As a result, it is now clear that serious errors may be introduced at every step between sample collection and final determination of analytes. Contamination of blood and other tissues with zinc and cadmium from rubber septa; chromium from stainless steel needles; and chromium, manganese, cobalt, nickel, molybdenum, and vanadium from forceps and cutting tools are a few of the well-documented examples (for examples, see references in Moyer *et al.* 1991). Contamination from these sources may be significant enough to be misinterpreted as exposure to toxic metals. Exhumed bodies present additional possibilities of contamination from soil, ground water, and embalming chemicals.

The most difficult contaminants to control are those toxic metals found at very low concentrations in tissues of interest, but high concentrations in contaminants. Samples containing levels of elements in the g/g or g/mL range may generally be handled

under normal clinical and analytical laboratory conditions. Elements present at ng/g or ng/mL levels or lower require class 100 or better clean room conditions, preleached sample containers, high-purity reagents, and special sample handling during analysis. Specific requirements must be set for each sample type and toxic metal. For complete multielement screens, the sample handling requirements are dictated by the element having the strictest requirements. Reagent, apparatus, and environmental control samples (blanks) should always be provided when possible.

(3) Selecting an Analytical Method. Choosing the best methods for sample preparation and analysis is perhaps the most difficult task in the quest for a complete screening procedure to detect toxic metal poisoning. No single analytical method has been shown to be effective for all toxic metals at physiologically relevant levels in a variety of tissues. The approach generally taken is to use multielement, semiquantitative screens since the goal of the analysis is to determine a large number of elements and, for most elements, the difference between background (unexposed) levels and levels resulting from intentional poisoning by toxic metals are quite large. Once the presence of high levels of a specific toxic metal is indicated, confirmation may be made by a well-accepted, single element method such as atomic absorption spectrophotometry (AAS).

Sample preparation such as desiccation, mineralization, or dissolution can be considered as a separate procedure from the analysis and must also be optimized. Required characteristics of a good sample preparation procedure are low levels of contamination, complete reduction of the sample matrix to a form compatible with the analytical method, and negligible loss of analytes. The best procedures for producing solid samples are mineralization by low-temperature oxygen plasma ashing and desiccation by freeze drying. High-temperature ashing has the advantage of requiring less equipment than the low-temperature methods and can be used for toxic metals of limited volatility. Microwave heating in a sealed Teflon container with strong oxidizing acids is rapidly becoming accepted as the best method for complete dissolution of tissue samples without loss of volatile analytes. Although slower, thermal heating with strong oxidizing acid mixtures is effective for many elements and tissue types.

Many analytical methods have been tried for full multielement screens of tissues with varying degrees of

success. Chromatographic and coulometric methods have had limited success in selected circumstances. Colorimetry, the Reinsch test, and silver mirrors have widespread historical use, but they lack sensitivity, specificity, and multielement capability compared to modern instrumental methods. However, these tests are still valuable for preliminary screening, particularly for stomach contents and urine samples in acute poisoning cases. A brief discussion of five instrumental analytical methods which have been used in the FBI Laboratory follows.

(a) X-ray Methods: X-ray methods, especially fluorescence (XRF), have found most applications as quick, nondestructive, semiquantitative screens in forensic and medical laboratories. Most x-ray procedures including both macro or micro techniques, use solid samples obtained by mineralization of tissue samples. Examples of XRF scans of two samples from poisoning cases are shown in Figures 1 and 2. Figure 1 shows a scan of a liver sample from a victim of chronic arsenic poisoning. The prominent arsenic lines are shown along with other characteristic elements observed in normal liver tissues. Figure 2 shows a scan of soup

which had been laced with thallium. The presence of the thallium is clearly indicated in the x-ray spectrum. Stomach contents of the victim showed similar thallium fluorescence peaks. The samples from these cases were spiked with indium as an internal standard and the organic portion was removed by low-temperature oxygen plasma ashing prior to analysis. The ash was then analyzed directly by placing the loose powder in the x-ray beam and recording the fluorescence spectrum. Electron beam induced x-ray fluorescence has the advantage of excellent spatial resolution which is useful for small inclusions of toxic metals in tissues. However, toxic levels of most elements in tissues cannot be detected by this method. There have been several recent reports of the use of particle induced x-ray emission for element detection in tissue samples, but the sensitivity for several important toxic metals is not adequate for a general screen. Total reflection XRF is a relatively new method which holds much promise for detection of toxic metal poisoning. As instruments become available in operational laboratories, their usefulness should be evaluated.

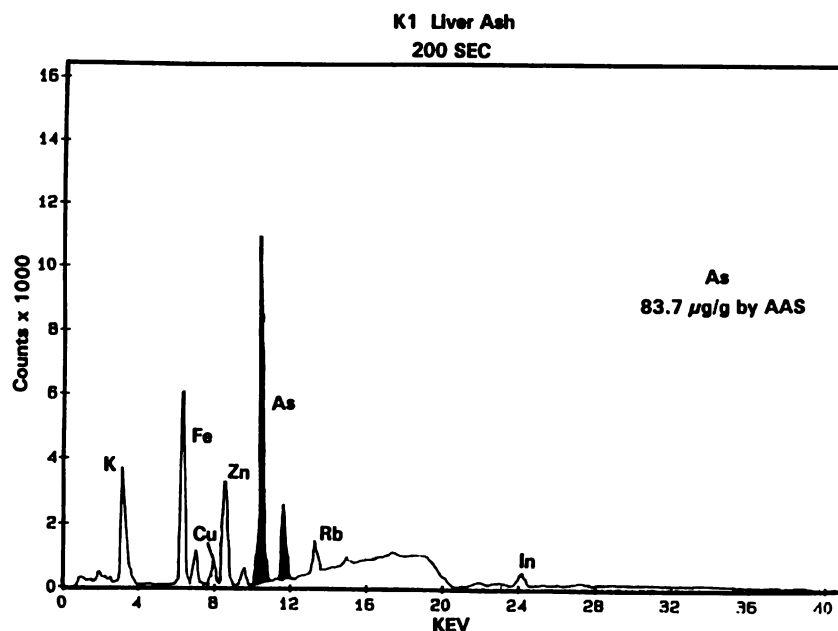


Figure 1. X-ray Fluorescence Spectrum of Ashed Liver Tissue from Victim of Chronic Arsenic Poisoning.

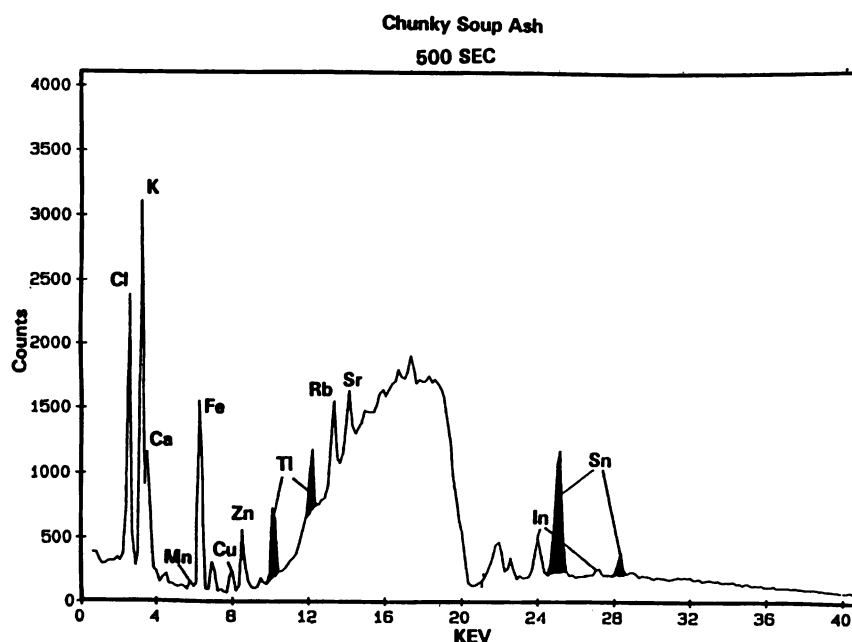


Figure 2. X-Ray Fluorescence Spectrum of Soup Ingested by Victim of Thallium Poisoning.

(b) Neutron Activation Analysis (NAA): NAA has been used for many years in the FBI Laboratory and has found the widest use in detection of As poisoning. Advantages of NAA include excellent sensitivity for several select elements, few spectral or chemical interferences, limited sample preparation, and excellent quantitation capabilities. Disadvantages of NAA include the limited availability of neutron sources, the need to handle radioactive materials, and the somewhat limited element coverage. NAA is not readily amenable to general element screens because of the wide ranges of nuclear characteristics and concentrations of the elements used in criminal poisonings.

(c) Atomic Emission Spectroscopy: Arc and spark instruments and more recently, inductively coupled plasma atomic emission instruments (ICP-AES) have been used for semiquantitative multielement screens. Most ICP-AES procedures require dissolution of samples and arc-spark methods require conversion of the sample to a dry powder, usually by dry ashing. Better quantitative multielement analysis, particularly when a limited number of elements is sought, is

obtained using ICP-AES with scanning monochromators whereby interelement interference corrections are more easily made. Detection limits for a number of significant elements are not good enough to detect biologically relevant levels in many tissues. Good reviews of ICP-AES methods for element measurements in tissue samples are published periodically in the *Journal of Analytical Atomic Spectrometry* (Taylor and Halls 1993).

(d) Mass Spectrometry (MS): Recent introduction of ICP-MS into forensic and medical laboratories has opened new possibilities for multielement screening of biological samples. Most applications to date have been research studies which have shown the feasibility of determining up to about 30 elements in biological tissues. Samples such as urine can be analyzed directly after dilution with water (Mulligan *et al* 1990). Table 1 shows toxic and normal levels and detection limits in urine with no sample preparation other than ten-fold dilution. Analysis of blood requires ion exchange or similar sample cleanup and possibly concentration. The determination of about 30 elements in tissues including surveys of samples taken at autopsies has been reported

(Lyon *et al* 1991; Vaughan *et al* 1991; Yoshinaga *et al* 1990).

(e) Atomic Absorption Spectrophotometry: AAS is not usually considered a multielement technique, but it is included here because it is the most widely available and well accepted method for confirmation of the presence of single elements found by the preceding screening methods. Analytical protocols are well established for most toxic metals and tissues. The sensitivity of AAS, particularly using furnace atomization, is adequate for most elements and interferences are generally well documented. Disadvantages of AAS are the need to dissolve samples and the slow speed of a single-element technique. AAS is becoming more applicable to limited multielement screens because some modern instruments provide rapid sequential determination of up to 12 elements. Less widely available, but also possible are continuum source AAS instruments with simultaneous multielement detectors based on diode arrays.

(4) Interpreting the Results. In order to detect the presence of toxic metals due to criminal poisoning, a normal level for each toxic metal must be obtained. A search of the literature to obtain these values is fraught with difficulties because a wide range of values is reported for many elements. Many of the reported element concentrations are incorrect or inappropriate because of errors in analysis, sampling, or population selection. The wide range between normal values and element

concentrations in tissues in fatal poisoning cases helps make this less of a problem than in clinical studies of low-level exposures. Newer analytical techniques provide excellent sensitivity and accuracy, but have yet to be applied to appropriate samples to give a database of reference values.

Correct detection of toxic metal poisoning can only be achieved when those responsible for sample collection, analysis, and data interpretation work together. The job of the analyst is made easier if the clinician can use symptomatic indications to limit the number of elements sought. The clinician must be familiar with the analytical and interpretive requirements of the examination to ensure appropriate collection and packaging of tissues. Contamination control and analytical quality assurance must also be a joint effort between clinical and analytical personnel.

AXIAL DISTRIBUTION OF TOXIC METALS IN HAIR

Human hair serves to a limited extent as an excretion unit for several toxic metals which are incorporated into the hair during its growth. The concentrations of at least 40 elements have been reported in human hair (Bencze 1990). For several elements, it has been demonstrated that their incorporation in the hair is correlated with their concentration in the blood at the time of hair formation. Thus, each hair stores chronological information concerning exposure to these elements over an extended period of time. For arsenic, in particular, hair is useful for assessing exposure, because hair arsenic concentrations reflect blood levels at the

Table 1. CONCENTRATIONS OF SELECTED TOXIC ELEMENTS AND ICP-MS DETECTION LIMITS FOR HUMAN URINE IN mg/L.

Element	Normal Level	Elevated Level	ICP-MS Det. Lim.
As	0.01 - 0.3	0.1 - 10	0.008
Cd	<0.0005	0.01 - 0.5	0.0005
Hg	0.002 - 0.01	0.1 - 10	0.003
Pb	0.01 - 0.1	0.1 - 0.3	0.001
Sb	<0.001	0.03 - 2	0.0002
Tl	<0.002	1 - 20	0.003

time of incorporation, arsenic has limited mobility once incorporated into hair keratin, and environmental contamination is generally negligible (Curry 1972).

Arsenic poisoning has a long history and arsenic probably remains the most frequently used toxic metal for intentional poisoning purposes. This arises in a large part because of the ready availability of various arsenic compounds in herbicide, rodenticide, and pesticide mixtures. In cases of intentional arsenic poisoning, it is desirable to be able to distinguish between chronic (long-term) and acute (short-term) ingestion because chronic exposure supports a charge of premeditated action. The axial distribution of arsenic in hair combined with an estimate of the growth rate of the hair allows the dates of ingestion to be inferred.

Several analytical methods have been used to determine axial arsenic concentration profiles in bundled hair samples, including the Gutzeit test, x-ray methods, AAS, ICP-AES, ICP-MS, and NAA (Berman 1980; Seta

et al 1988). In the FBI Laboratory NAA has been used successfully to analyze 1 cm increments of bundled hair samples for over 20 years. NAA results for arsenic concentrations as a function of axial position in hairs from several subjects in criminal cases are shown in Figures 3 - 5. For purposes of comparing these results, we can assume that the hair growth rate is about 1 cm/month and that levels of arsenic in the hair of unexposed individuals are less than 1 g/g.

The first case, shown in Figure 3 represents a typical axial profile resulting from chronic high-level arsenic exposure. In this case, the victim was the third husband of a woman who apparently poisoned her previous two husbands by putting arsenic in their food. The victim had a long period of off and on hospitalization prior to his death. The hair sample was collected following the victim's death and shows a pattern of continued high-level exposure to arsenic over a period of several months.

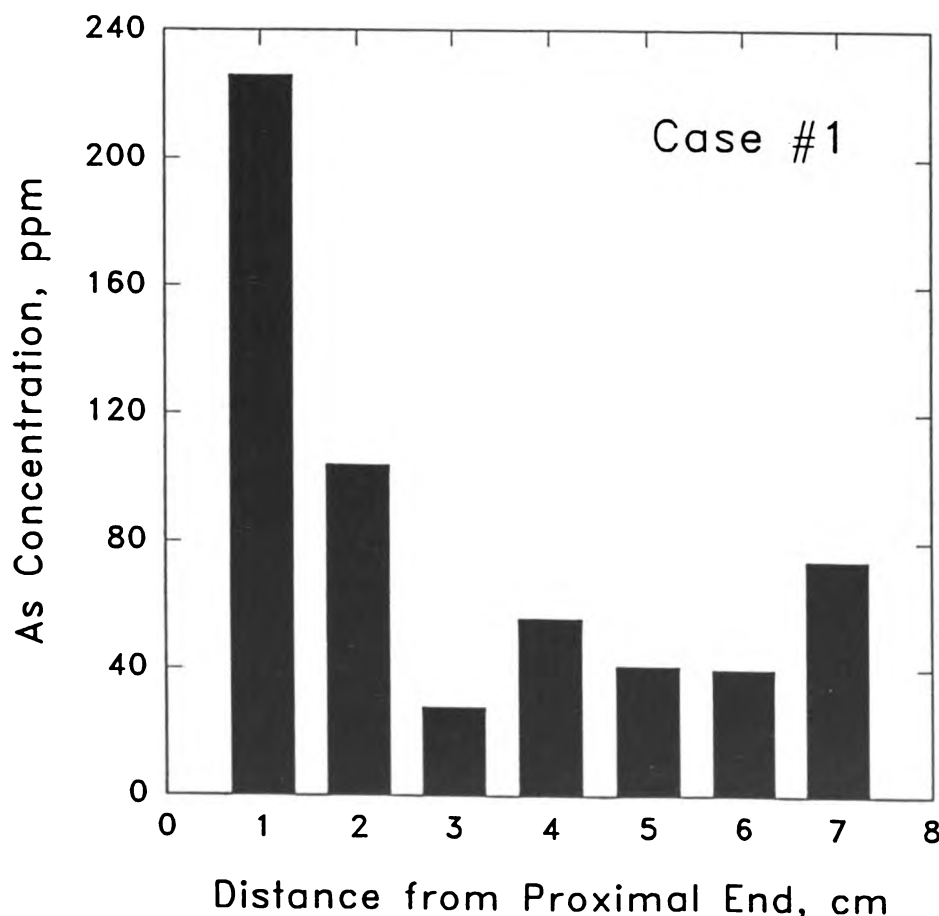


Figure 3. Axial Distribution of Arsenic in Hair from Chronic Poisoning Victim. Concentrations Shown are $\mu\text{g/g}$ in Hair Taken at 1 cm Increments Starting at the Proximal End.

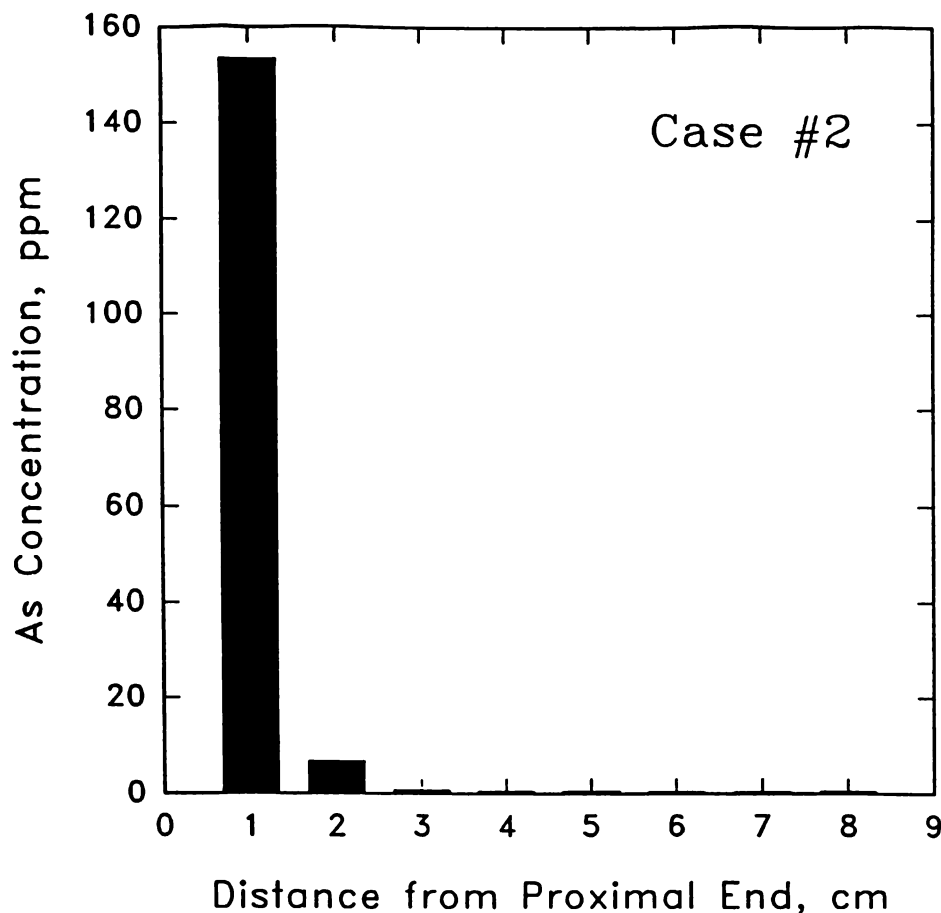


Figure 4. Axial Distribution of Arsenic in Hair from Acute Poisoning Victim. Concentrations are as in Figure 3.

A different arsenic profile is seen in a second case, shown in Figure 4. In this case, the subject became violently ill in late January, was hospitalized, and subsequently recovered. Urinary arsenic concentrations were 387 g/L on January 31 and 151 g/L on February 10. The victim's wife was suspected of poisoning her husband and hair samples were collected to assess whether exposure had been chronic or acute. As the profile indicates, the victim was subject to high levels of arsenic exposure in the couple of months preceding hospitalization.

Results of arsenic determination in three people in a third case are shown in Figure 5. In this case, a man became ill with symptoms of toxic metal poisoning and was diagnosed with arsenic poisoning in the hospital. He subsequently recovered. To determine whether environmental exposure in his home could have been the source of the arsenic, a sample of his wife's hair was also collected. Upon further investigation, it was determined that a previous husband of the woman had died under suspicious circumstances several years previously. The deceased former husband was disinterred and samples of his hair were collected. The results of analysis of the hair from the three subjects in this case

are shown in Figure 5. The arsenic profiles indicate that both males were exposed to high levels of arsenic over extended periods of time and the female's hair arsenic levels were in the normal range.

Recently, a method was reported for accurate measurements of arsenic distributions along a single hair using 5 mm portions of hair placed directly into a graphite furnace AAS as solid samples (Koons and Peters 1993). Advantages to this analytical method include its speed, low contamination, and good sensitivity and accuracy. The use of single hairs rather than bulk hair samples reduces potential problems in interpretation of results arising from averaging hair to hair variation in arsenic distribution. There is considerable variability in both growth rates and arsenic accumulation among individual hairs from a victim's head. As a result, methods which require multiple hairs (aligned and bundled together) show peak As concentrations spread out over a longer time span than the actual exposure. Similar difficulties arise when screens for As poisoning are done using bulk hair samples. In cases of acute exposure, the narrow bands of those hairs containing high As levels are diluted by the large mass of unexposed hair with the result that excessive exposures may be

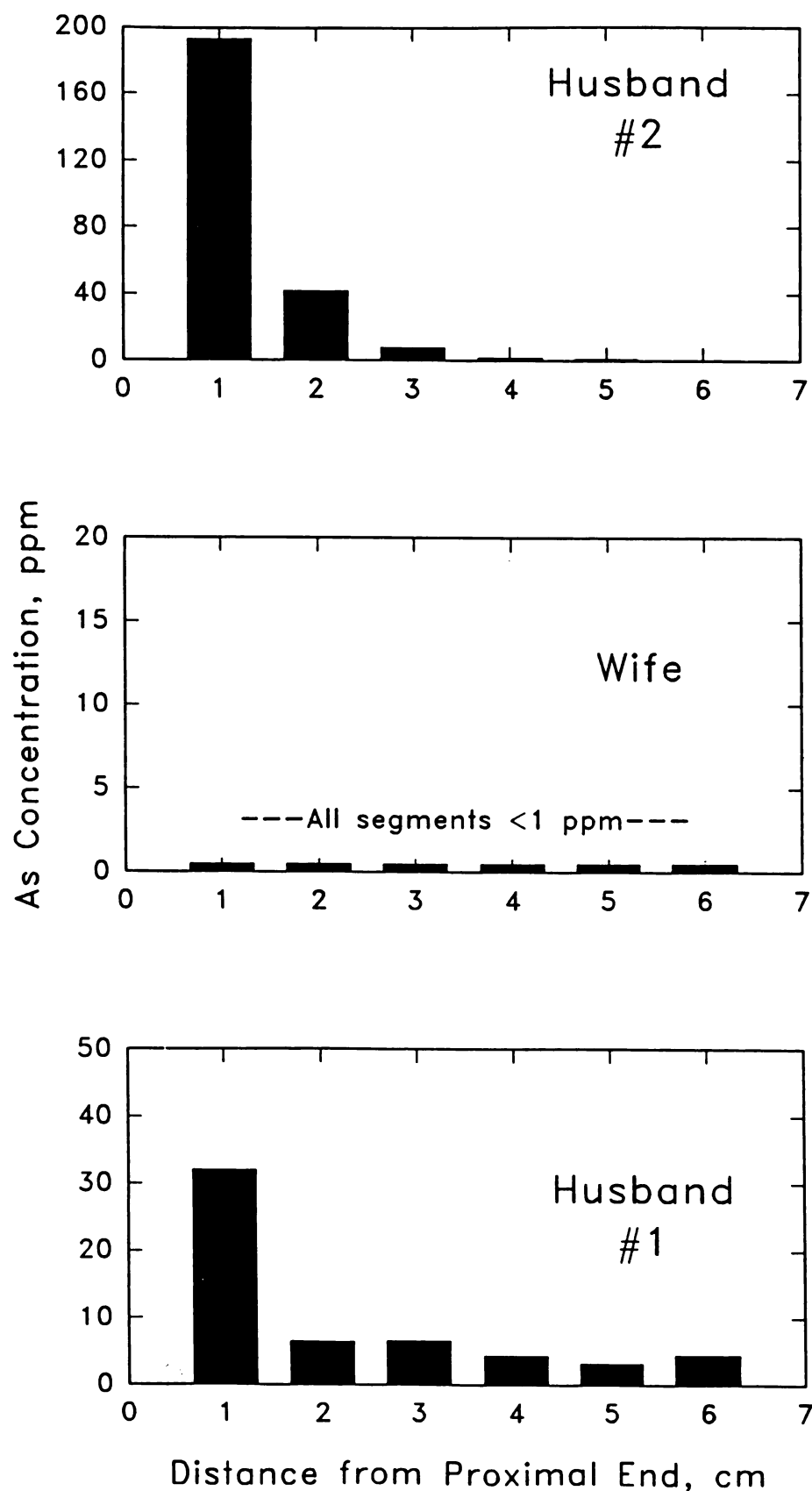


Figure 5. Axial Distribution of Arsenic in Hair of Three Subjects in Case #3. Concentrations Are as in Figure 3. Note the Different Concentration Axis Scales for the Three Subjects.

missed altogether. The literature values to date for arsenic concentrations in hair from poisoning victims were derived from bulk or aligned multiple hair samples, so they are consistently lower than peak arsenic concentrations determined using single hairs.

The development of new analytical methods is continually improving our ability to accurately measure lower and lower concentrations of toxic metals. As the analytical technology develops, great care must be taken to analyze appropriate tissue samples from well-categorized subjects to provide baseline data needed for the recognition of toxic metal poisoning. Advances in analytical methodology will improve our ability to detect toxic metal poisonings only if clinical and analytical professionals work together to obtain appropriate data.

ACKNOWLEDGMENTS

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Volatile Substance Abuse (Solvent Abuse)

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The ability of volatile compounds to affect consciousness has been recognized for many years. Nitrous oxide was discovered in 1776 by Sir Joseph Priestly and synthesized the same year by Humphrey Davy. In 1800 Humphrey Davy, then 21 years old and superintendent of the Medical Pneumatic Institution in Bristol, published his book on nitrous oxide in which he noted that it might be used during surgical operations. This observation was ignored for 46 years, probably because of religious objections to pain relief.

"In pain you shall bring forth children."
(Genesis 3:15)

Figure 1 shows the title page of Davy's book; Figure 2 shows a poster advertising a demonstration.

"...as nitrous oxide in its extensive operations appears capable of destroying physical pain it may probably be used with advantage during surgical operations." (Davy 1800)

At nitrous parties, Davy along with notables such as the poets Samuel Taylor Coleridge and Robert Southey, and Peter Roget (of thesaurus fame) experienced the lightheaded euphoria and uncontrollable fits of laughter resulting, hence the name laughing gas.

The discovery of anesthesia has been attributed to Horace Wells, a dentist, who in 1846 observed a man intoxicated with nitrous oxide at a public demonstration injure his shin and feel no pain (Duncom 1947). The following day he inhaled nitrous oxide and had a wisdom

tooth removed painlessly by a student. Nitrous oxide anesthesia was unsuccessfully demonstrated at Massachusetts General Hospital in 1846 (the dose was probably insufficient), but in the following year an operation was successfully performed using ether.

During so-called "ether frolics" people would consume ether as a liqueur, sometimes eating strawberries dipped in it. Ether had also been prescribed as a medicinal agent often to be consumed as the liquid. Inhalation of ether for entertainment was commonplace around 1839 amongst medical students in the United States, and there are reports of dentistry and minor surgery performed under ether in 1842.

"Dr. Miller calls attention in the *Western Journal* to the pernicious effects of the inhalation of this (ether) vapour—a habit which seems to prevail amongst young persons in some districts of the United States. He refers to three cases, one of which terminated fatally..."

On January 19, 1847, St. George's Hospital in London invited John Snow to administer ether to patients undergoing major surgical operations, thus becoming the first hospital to appoint an official anesthetist. He went on to collect data on mortality from the use of anesthetics and collected data on 48 deaths from chloroform, until his untimely death in 1858 (Snow 1858). His epidemiological work on the mode of communication of cholera in 1848 is well known; he deduced it was transmitted by drinking water.

Chloroform was discovered in 1831 virtually simultaneously in Germany, France, and the United States; accounts of its misuse in the United States appears

RESEARCHES,
CHEMICAL AND PHILOSOPHICAL;
CHIEFLY CONCERNING
NITROUS OXIDE,
OR
DEPHLOGISTICATED NITROUS AIR,
AND ITS
RESPIRATION.

By HUMPHRY DAVY,
SUPERINTENDENT OF THE MEDICAL PNEUMATIC
INSTITUTION.

LONDON:
PRINTED FOR J. JOHNSON, ST. PAUL'S CHURCH-YARD.
BY BIGGS AND COTTELL, BRISTOL.
1800.

Figure 1.

the same year. In November 1847 in Edinburgh, Sir James Simpson, an obstetrician, and his associates inhaled chloroform and immediately began using it in obstetric practice. Simpson refuted religious objection to pain relief by declaring that God used an anesthetic while removing Adam's rib to create Eve.

"So the Lord God caused a deep sleep to fall upon the man, and while he slept took one of his ribs..."

On November 13, 1847, the day following Simpson's first use of chloroform, an advertisement was placed in *The Scotsman* by a local pharmacist promoting its sale to the public. Anesthesia finally became respectable when Queen Victoria accepted the use of chloroform, administered by John Snow on April 7, 1853, during the birth of her eighth child, Prince Leopold. Developments were rapid, but it was quickly noted that chloroform could cause sudden cardiac deaths (Simpson 1858).

A GRAND
EXHIBITION
OF THE EFFECTS PRODUCED BY INHALING
NITROUS OXIDE, EXHILERATING. OR
LAUGHING GAS!
WILL BE GIVEN AT *The Theatre Royal*

Monday EVENING, 18th 1846.

50 GALLONS OF GAS will be prepared and administered to all in the audience who desire to inhale it.

MEN will be invited from the audience, to protect those under the influence of the Gas from injuring themselves or others. This course is adopted that no apprehension of danger may be entertained. Probably no one will attempt to fight.

THE EFFECT OF THE GAS is to make those who inhale it, retire

LAUGH, SING, DANCE, SPEAK OR FIGHT, &c. &c.

according to the leading trait of their character. They seem to retain consciousness enough not to say or do that which they would have occasion to regret.

N. B. The Gas will be administered only to gentlemen of the first respectability. The object is to make the entertainment in every respect, a genteel affair.

For a full account of the effect produced upon some of the most distinguished men at Europe, see Hager's Medical Dictionary, under the head of Nitrogen.
The History and properties of the Gas will be explained on Monday.

Figure 2.

By the 1960s there were numerous reports from all over the world of the abuse of solvent-containing commercial products, mostly glue and solvents from paint thinners. The problem became known as glue sniffing or solvent abuse. By 1967 the wider problem was recognized. Press and Done (1967) in a review article discussed sudden death from toluene, butane, trichloroethane, and aerosol propellants. Because now all the products abused are not glues and all the substances misused are not solvents, the term volatile substance abuse (VSA) or possibly inhalant abuse (although this could be thought to include snorting cocaine) is more accurate.

There is extensive international literature, which consists mainly of isolated case reports, reports of chronic toxicity, and a few prevalence studies and reviews. There are no systematic studies of morality apart from our own (Anderson *et al.* 1985). Reliable prevalence data is hard to come by and is probably not very enduring

because VSA seems to come and go and change its character rapidly at any particular locality. Ives (1991) in a publication of the UK National Children's Bureau, reviews prevalence studies and concludes that comparison between studies is difficult because of methodological differences. However, he concludes regional differences are apparent and, despite varying media attention, the problem remains. Sex differences in the incidence of the practice are unclear with males probably exceeding females, but not by much. This uncertainty makes hazard assessment difficult because, although we can measure mortality, it is impossible to access the population at risk (the denominator problem).

The problem is often thought to affect principally disadvantaged teenagers from deprived urban areas, but there is evidence that it involves a much wider group. Very different users are involved, ranging from the young experimenter who tries it a few times then gives up, through the hardened user in his early 20s, to the middle-aged man who uses volatile compounds to augment sexual pleasure, often combined with partial asphyxia. However, in most cases VSA is merely a transient act of experimentation with only a minority of experimenters going on to sniff regularly and even fewer becoming in any way dependant. Chronic users are often considered to be very low in the hierarchy (social order) of drug abuse and may be considered deviant even by other drug users.

Why then do people abuse volatile compounds?

- enjoyable, produces pleasant sensations
- rapid onset and recovery (if not chronic)
- readily available
- their peers do it
- cheap, free if stolen
- legal, if not stolen
- accessible to young people before alcohol
- no hangover?

It is often difficult for adults to appreciate that VSA is enjoyable, but many young users enjoy it sufficiently to repeat the experience. Some claim to

experience and enjoy pseudo-hallucinations, they see and hear things that are not real, but know that they are not. Many of the characteristics of VSA are very convenient for a young person. A cheap or free "high" of such short duration, which is possible to indulge in after school and still return home sober, is a convenient and attractive escape for many youngsters.

The acute effects are dose dependant; the pleasant desired effects achieved at moderate doses are:

- excitement, euphoria, exhilaration
- pseudo-hallucinations (visual and auditory)

The less-desirable effects with attendant risks come with a higher dose are:

- cerebral depression
disorientation, blurred vision,
dizziness, slurred speech, ataxia, and
drowsiness
- unconsciousness
- death

The risk of sudden cardiac death appears unrelated to the frequency of use except that inexperienced users may be more inclined to overdose. VSA is unlike almost any other form of intoxicant use in that there is no intuitive unit dose. With alcohol or tobacco the unit dose is obvious; even with heroin, cocaine, or cannabis some measure of dose is implied by the amount sold at the street level. By contrast it is difficult for the naive user to estimate the required dose of a fire extinguisher or a cigarette lighter refill. It is also difficult for the experimenter to monitor the dose as he becomes more intoxicated and with the gaseous products inhaled from a bag, the possibility of anoxia is a complicating factor.

PRODUCTS AND THE SOLVENTS THEY CONTAIN

It is convenient to divide the products misused into four groups: adhesives, aerosols, fuel gases, and solvents, and to consider the range of volatile compounds within each group. The boiling point is important because it affects the ease with which the product may be abused and also the maximum possible concentration. For example, the vapor from a product containing a

higher boiling point solvent such as toluene even when concentrated in a bag will contain a mixture of air and vapor, whereas, a bag inflated with butane (a gas) could contain no air.

Adhesives

Not all adhesives contain organic solvents, but those that do are readily available in most developed communities. Common amongst them are contact adhesives (used for laying floor tiles, carpeting, or fixing laminate to work surfaces). Other adhesives which youngsters have legitimate access to also contain solvents, such as those for bicycle inner tube puncture repair and model airplane cement. Toluene is believed to be the compound responsible for the intoxication from most adhesives, because we detect it in the blood of most glue sniffers. However, many other volatile compounds are also present in adhesives and may contribute to chronic toxicity.

Solvents in Adhesives	
Compound	Boiling Point °C
Toluene	111
Xylene	145
Acetone	57
Butanone	80
Hexane	69
Ethyl Acetate	77

Aerosols

In order to understand aerosol misuse it is necessary to understand how aerosols work. A typical aerosol product consists of a metal (tin plate or aluminum alloy) pressure vessel with a valve. The active principle (e.g., in the case of a deodorant, aluminum chlorhydrate) is dissolved or suspended in a solvent system and the propellant, a low-boiling liquid or gas, provides the energy source to expel the product in a finely divided form (aerosol) (Figure 3).

It is the propellant which is abused, so that almost any aerosol can be misused. However, it is more convenient (easier) to misuse products which contain a high proportion of propellant (deodorant, antiperspirant, or fly spray) rather than those containing little (shaving foam). Some products which only contain propellants are particularly attractive, such as pain relief sprays and products for blowing dust from photographic equipment or chilling electronic components. The propellant can often be released free from the product by inverting the can, but it is possible to use a valve (essential with compressed gas products) that prevents this.

Most aerosols have been reformulated recently. The so-called ozone friendly or ozone benign compounds have been substituted for the chlorofluorocarbon (CFC) propellants which were held responsible for depleting the ozone layer. The British Aerosol Manufacturers Trade Association claim that over 90% of British aerosols

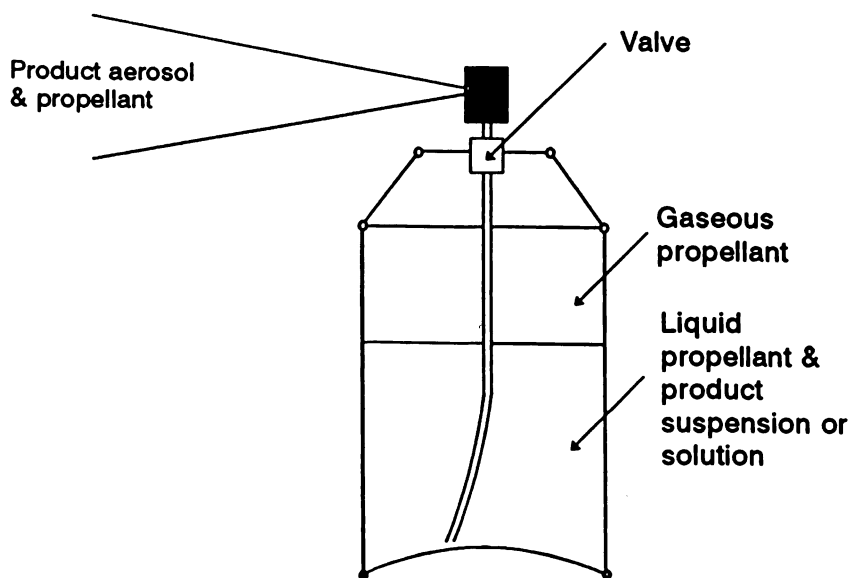


Figure 3.

are CFC-free now. The short-term solution adopted has been to use deodorized butane, which carries with it a fire hazard. Some medical products retain CFCs and some other products use FC 22 which is thought to cause less ozone depletion. Other European countries use dimethyl ether, sometimes blended with FC 22 to form a nonflammable azeotrope. A new compound, FC 134A, is in production primarily as a refrigerant, but may be used in aerosols if expense permits. New technology such as pumped sprays, compressed gas (nitrogen or air), or novel systems using metal hydrides may gain acceptance if cost can be reduced.

The Revised Montreal Protocol: London, June 1990, which is due to come into force on January 1, 1993, and is mandatory for all parties, gives January 1, 2000, as the latest date by which most CFCs should be phased out. However, the European community has agreed removal by June 1997.

Compounds in Aerosols	
Compound	Boiling Point °C
Ozone Hostile	
FC 11	24
FC 12	-30
Dichloromethane	40
FC 114	4
Ozone Friendly	
FC 22	-41
Ozone Benign	
n-Butane	-1
Iso-Butane	-12
Dimethyl Ether	-25
FC 134A	-27

Fire Extinguishers

Vaporizing liquid fire extinguishers, although not strictly aerosols, are conveniently considered here because they contain chemicals with very similar physical and chemical properties to aerosols. Modern extinguishers for use in transportation contain bromochlorodifluoromethane sometimes modified with other fluorocarbons to increase the pressure. Fire extinguishers have to be in view and accessible and, consequently, are vulnerable to theft (one London bus depot at the height of an epidemic was spending £ 2,000 per month replacing them). Computer rooms are often protected by fixed installations containing bromofluorocarbons which are

unlikely to be abused because of restricted access. Carbon tetrachloride was a common firefighting agent in World War II under the name Pyrene®, but is now obsolete and bromomethane was used in aircraft. The Montreal Protocol allows the continued manufacture of some bromofluorocarbons for safety critical applications, but many will probably be replaced with carbon dioxide.

Compounds in Fire Extinguishers	
Compound	Boiling Point °C
Carbon Tetrachloride	77
Bromomethane	4
Bromochlorodifluoromethane (BCF)	-3
Bromotrifluoromethane	-58

Fuels

Portable sources of fuel are commonly used to power domestic products such as camping stoves, brazing torches, paint strippers, and hair stylers. Cigarette lighter refills are also readily available and are very attractive to misuse. Most of these will contain fuel grade butane in disposable canisters very much like aerosols. Professional equipment often uses propane which, because of its lower boiling point (higher vapor pressure), has to be stored in more substantial and consequently refillable containers.

Gases	
Compound	Boiling Point °C
Methane	-161
Propane	-42
n-Butane	-1
Iso-Butane	-12

Liquids: Gasoline (petrol) is freely available in almost all countries and may be abused, with the risk of lead poisoning (Rischbieth *et al.* 1987) and hexane neuropathy (Hall *et al.* 1986). It seems that petrol is ignored despite being readily accessible if other compounds (products) are available. This leads us to speculate that the high obtained is probably of a lesser quality. Paraffin, diesel oil, and white spirit are insufficiently volatile to be misused.

Solvents

The most common domestic source of bulk solvents are dry cleaning or spot-removing products and typewriter correction fluid thinner. Tipp-Ex®, a leading brand of correction fluid in the UK has been forced to develop a nonsolvent-containing product to retain its significant market share when faced with a ban in schools. Some paint, paint thinners, and paint strippers also contain abusable solvents, but this is not a significant problem in the UK.

Carbon tetrachloride is widely recognized as being hepatotoxic and is not generally available unless stolen from laboratories. The Montreal Protocol requires the replacement of carbon tetrachloride by the year 2000 (European Community, January 1998) and 1,1,1-trichloroethane by January 2005.

Compounds Used as Solvents	
Compound	Boiling Point °C
Carbon Tetrachloride	77
1,1,1-Trichloroethane	74
Trichloroethylene	87
Tetrachloroethylene	121
FC 113	
1,1,2-Trichlorotrifluoroethane	48
Dichloromethane	40

Anesthetics

Although these compounds are not freely available, they are misused by professionals who have access to them. Nitrous oxide intended for anesthesia has been misused by dentists (Fraunfelder 1988). Most hospital operating theaters count bottles of liquid anesthetic before and after an operating session to prevent pilfering. There is one use of nitrous oxide which makes it available in the community of the UK. It is the only gas at present approved for food use and consequently is found as the propellant in aerosol cans of whipped cream. There are domestic devices available to make whipped cream in this way at home (Sparklets®, British Oxygen Company), and consequently it is possible to buy small bulbs containing nitrous oxide in chemists shops (drug stores). These have been misused and have even caused a fatality when connected to a stolen anesthetic apparatus.

The topical anesthetic ethyl chloride is misused in some countries during carnivals when it may be squirted on people or sniffed from the glass bulb in which it is usually supplied.

Compounds Used as Anesthetics	
Compound	Boiling Point °C
Nitrous Oxide	-88
Diethyl Ether	35
Chloroform (Obsolete)	61
Halothane	50
Enflurane	57
Methoxyflurane	105
Isoflurane	49
Cyclopropane	-33
Trichloroethylene (Obsolete)	87
Topical Ethyl Chloride	12

Effects and Mode of Action

Small inexpensive products from which it is easy to extract the volatile component in a respirable form are preferred. A typical example of an ideal product is a cigarette lighter refill containing butane. A similar product also containing butane such as Camping Gas® is not commonly misused because it does not have a ready means of extracting the gas (valve), but requires the cylinder to be connected to an appliance.

The hazard of butane misuse is considerably greater if the gas is inhaled directly from the cylinder by clenching the nozzle of a cigarette lighter refill between the teeth, with the attendant risk of inhaling gas (or liquid) cooled to -60°C by expansion. Fuel grade butane may also contain butene (1,3-butadiene) which is carcinogenic.

The increasing popularity of butane is probably because of a combination of the quality of the experience, short duration of action, and the accessibility and convenience of the commercial presentations. Fashion and peer group pressure are also important factors.

Features that make a domestic product attractive to abuse:

- contain suitable volatile substance
- readily available (accessible)
- clean (unlike glue)
- cheap or easily stolen
- ready access to the volatile component
- portable and easily concealed
- legitimate for young person to possess
- brand loyalty, inviting name (Soft & Gentle® hair spray)?
- no adverse labeling?

VSA is usually characterized by a rapid onset of intoxication and a rapid recovery, but experienced users can sustain a "high" by repeatedly sniffing. The intoxication is not unlike that produced by ethanol, but high doses may produce frightening hallucinations. Tinnitus ataxia and agitation may precede changes of perception, dangerous delusions such as those of being able to fly and the feelings of invulnerability (Evans and Raistrick 1987). Nausea and vomiting with the risk of aspiration may occur. Sudden death may result from trauma, asphyxia, cardiac arrhythmia, or rarely vagal stimulation leading to bradycardia (Shepherd 1989). Sudden fright, exertion, or sexual activity may precipitate an arrhythmia since the compounds sensitize the heart to catecholamines.

The acute mode of action of these compounds is not well understood, but is determined more by the physical than the chemical properties. The rapid effects and recovery tends to suggest a physical (or weak chemical, hydrogen bonding) effect rather than covalent chemical bonding to a receptor. The risk of chronic organ damage is, however, often associated with metabolites and consequently, the chemistry. It is the nature of the product as much as the physical properties of the volatile compound that determines the misuse potential and the acute risk.

Epidemiology

There is very little reliable information about the scale of the practice in the UK. The problem seems to come and go at any one location, but anecdotal information gleaned from youth workers suggests it never disappears completely. It is difficult to propose methodology to monitor the incidence of the practice of a problem of this nature. However, it is more straightforward to systematically monitor deaths, but without the denominator of the size of the problem (for different products, compounds, and practices) it is not possible to assess the hazard. John Snow had the same problems with his anesthetic deaths in 1858, he had no idea how many anesthetics were administered.

We have collected data since 1981 on morality from VSA from all over Great Britain and Northern Ireland. We became aware that there was an underreporting of the problem when our laboratory alone investigated more deaths than were shown by the official statistics. The problem was that in the absence of an International Classification of Diseases (ICD) code for VSA, deaths were distributed amongst many categories. We decided to use a press-cutting agency, which reads all local and national newspapers, to send us clippings on all reports of deaths from VSA, and consequently, alert us that a death had occurred. In Britain, Coroner's inquests are open hearings which are used by the local press as training grounds for junior reporters. The death of a young person, particularly in tragic circumstances, is always regarded as being newsworthy and is always reported, at least locally. We use this notification to alert us that a death has occurred and then we write to the Coroner to request details of the death, including a transcription of the evidence and a copy of the pathology and toxicology reports. We also write regularly to every Coroner (158) in England and Wales and the Crown Office in Scotland to ensure we do not miss any (Figure 4).

We are fortunate that death certificates are public documents in Britain so that the Office of Population Censuses and Surveys, Medical Statistics Division, can provide us with copies of them for England and the General Register Office can provide them for Scotland. In Northern Ireland, the Lord Chancellor's Department provides information on deaths and the General Register Office for Northern Ireland provides the death

Data Collection & Dissemination

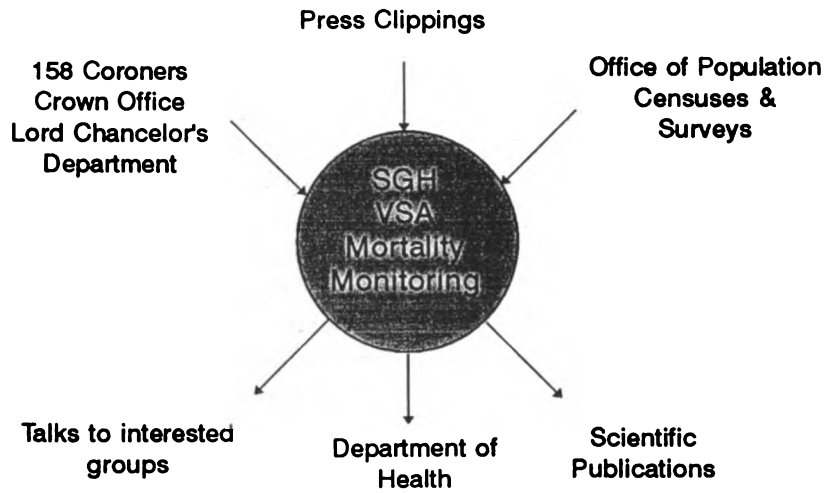


Figure 4.

VSA DEATHS BY YEAR (n=1113)

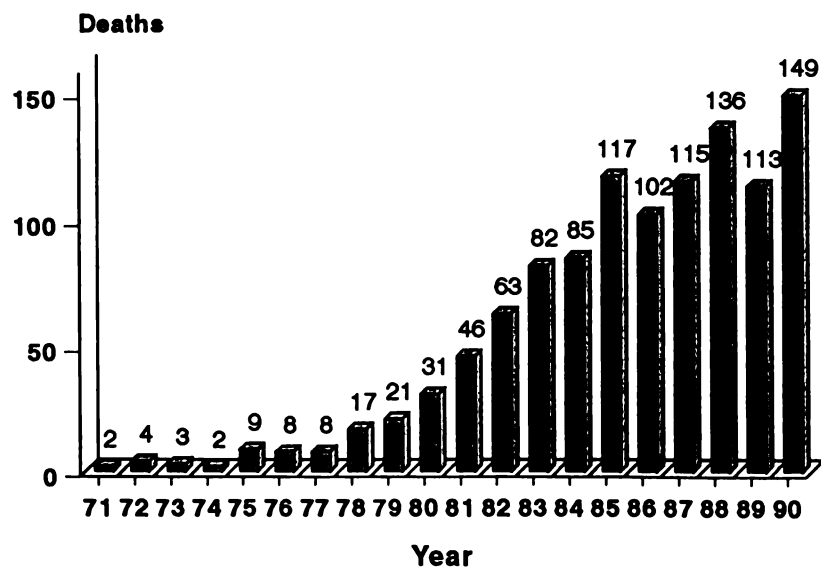


Figure 5.

certificates. The Deputy Viscount in Jersey and HM Greffier in Guernsey enable us to include information for the Channel Isles. In most other European countries, death certificates are confidential documents.

The ICD code 304 (drug dependence) now has a subdivision 304.6 (other) which includes glue sniffing and rather idiosyncratically Absinthe addition, it also specifically excludes tobacco dependence. In practice this seems to be used for all forms of VSA. The Office of Population Censuses and Surveys also operates a supplementary digit coding system where certain deaths are coded by hand and extracted for us.

We have data on 1,113 deaths from 1971 to the end of 1990, the last year for which we have complete data. During 1990, there were 149 deaths (the highest number ever recorded in 1 year). Figure 5 gives the total number of deaths for each year. The annual number of deaths has been steadily increasing since the early eighties, and although this is not a constant trend (from 1983 to 1990), this is equivalent on average to an increase of 8.9% per year.

These deaths comprise an important proportion of all deaths in young people as death in this age group is rare. In the UK the mortality is approximately equivalent to that from asthma, but significantly less than that from road traffic accidents (Figure 6). It is

also much higher than that from all other forms of drug abuse in this age group.

The most common age at death is 15 or 16 (Figure 7) with those occurring in the 14 to 18 range accounting for 62.4% of all VSA deaths from 1971 to 1990; 73.4% of those who died were under the age of 20. The youngest death is a female aged 9 years. Prevalence studies tend to suggest roughly equal numbers of males and females abuse volatile substances, but 87% of 1990 deaths and 88% of all deaths occurred in males. It seems to be a problem affecting predominately white children. Social class is difficult to assess in young people, but we believe deaths occur in all classes.

Figure 8 shows the number of deaths by primary substance abused. There was very little change in 1990 in the substances abused compared with previous years. Proportionately, gas fuel deaths appear to have decreased slightly since 1989, while the proportion of deaths due to other substances has increased. This is largely due to the marked increase in reported deaths linked to the inhalation of the contents of fire extinguishers: 16 in 1990, against 3 for the previous year, and a total of 24 for 1971 - 1988 (Figure 9). Early indications are that the number of deaths involving fire extinguishers in 1991 will be closer to the established pattern. The use of bromochlorofluorocarbons in fire extinguishers may be phased out under the Montreal Protocol.

Deaths England & Wales 1990

	Male & Female
Age 10-14	
Volatile Substance Abuse	24
Road Traffic Accidents	129
All causes	568
Age 15-19	
Volatile Substance Abuse	94
Road Traffic Accidents	635
All causes	1767

OPCS DH2 No17, E810-819, Table 2

Figure 6.

Deaths 9-19 years

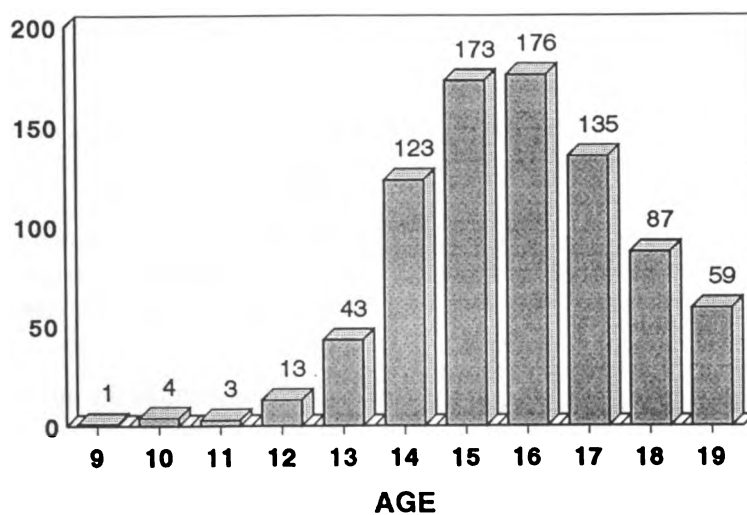


Figure 7.

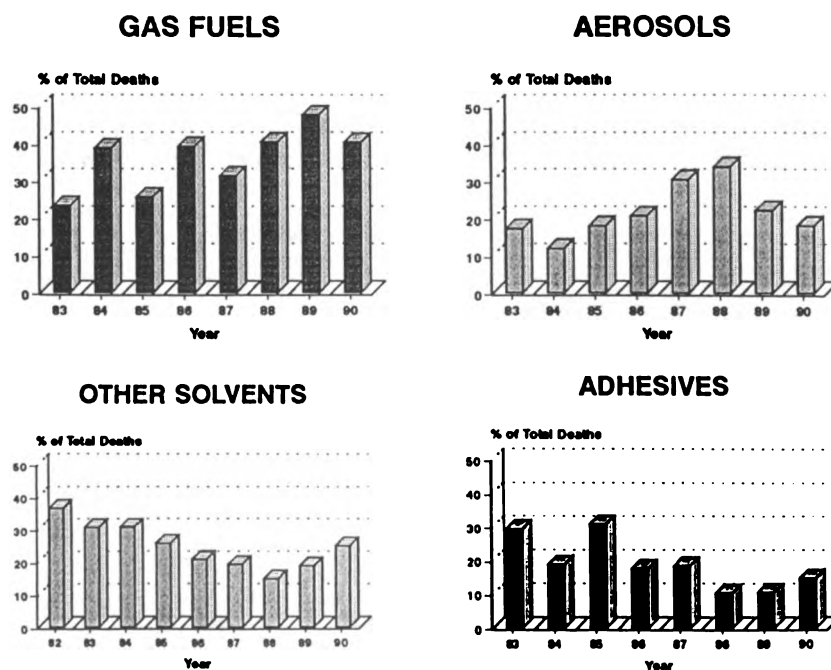


Figure 8.

In approximately 4% of deaths, more than one substance had been abused in the period immediately preceding death. In 1990 gas fuels were involved in approximately 40% of deaths, aerosols in 20%, glue in 17%, and other substances in 27%.

Since 1971 gas cigarette lighter fuel has been linked to 80.4% of gas fuel deaths; 27.9% of all deaths. Deodorants and antiperspirants were involved in 46.3% of deaths in the aerosol group, or 9.5% of all deaths.

In 1990 in 103 cases (69.1%), there was evidence of a previous history of solvent abuse. The remaining 46 (30.9%) either died on the first occasion, or one of the first occasions, or there was no evidence of their ever having indulged in VSA before. There is no evidence that long-term sniffing makes a subject tolerant. Conversely, there is no evidence that a history of sniffing increases the risk of sudden death.

We code the place where the episode of VSA associated with death took place as well as the place of death. Figure 10 shows where substances were abused. In 48% of deaths the substance was abused in a public place such as a park, shopping center, or the street and 48% in the home of a friend or residential institution. We particularly exclude data on deaths occurring in the workplace which is collected in an ad hoc fashion by the Health and Safety Executive.

The cause of deaths have not changed markedly over the years except that the involvement of plastic bags seems to have decreased, possibly because of publicity and the decline in the use of glue (Figure 11). Death is often attributed to a cardiac mechanism, but there is little positive evidence of this at postmortem. Laboratory investigations were performed in 142 of the 149 deaths in 1990.

Laboratory Services

VSA is a sufficiently common cause of death in young people to make it advisable to screen all deaths, under 25 years old, for the presence of volatile compounds. Similarly, the involvement of VSA should be considered in the investigation of the acute presentation of young people to accident and emergency departments. Psychologically disturbed and behaviorally maladjusted teenagers should also be investigated. Epidemiological studies of the consequences of VSA can be greatly strengthened by the appropriate use of toxicological analysis. In our hands, it is almost always possible to confirm the suspicion of VSA by analysis of a blood sample, even under far from ideal circumstances (for example, poor sample collection or the sample delayed in the post).

The selection of the biological fluid and the compound targeted for analysis needs to be appropriate

Deaths from Fire Extinguishers BCF (Bromochlorodifluoromethane)

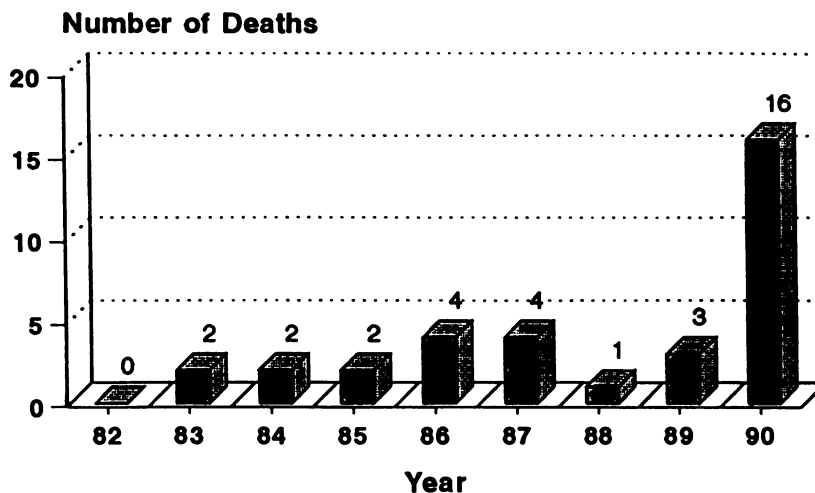


Figure 9.

Where substance was abused (1990)

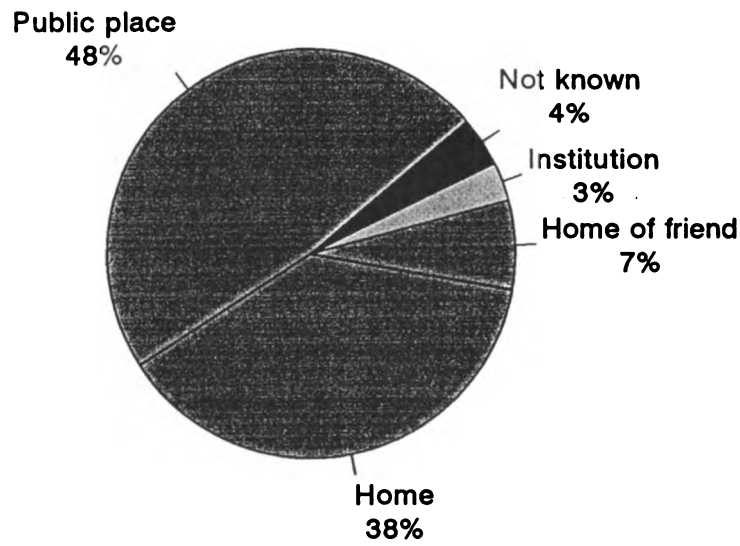


Figure 10.

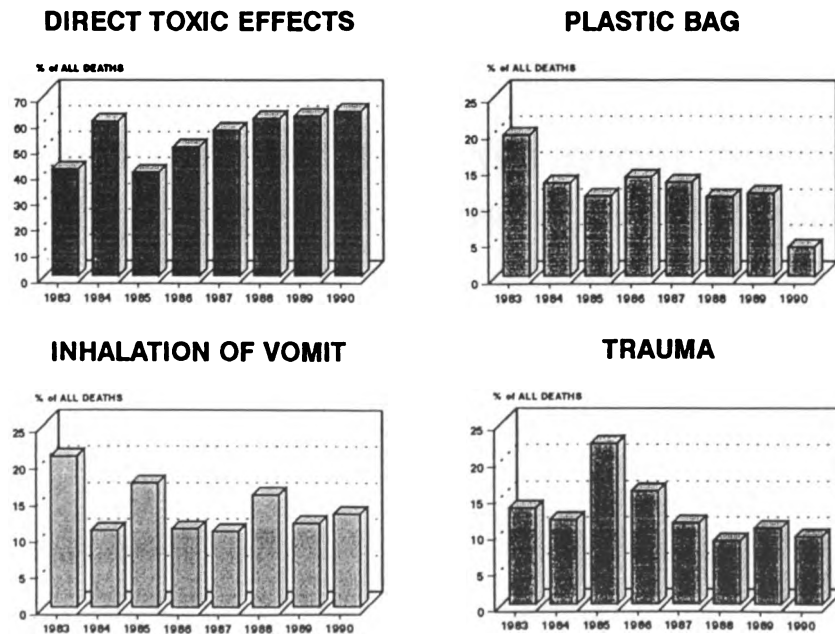


Figure 11.

for the task at hand. The analysis of blood from the unchanged volatile compound is the strategy of choice when investigating the possibility of current intoxication and at autopsy or when invasive sampling is acceptable. Analysis of breath for the unchanged compound is valuable when investigating current intoxication or past exposure when a noninvasive procedure is required. Well-perfused fatty tissues (brain) often yield higher concentrations of volatile compounds and are valuable when investigating suspected exposure to complex mixtures such as petroleum distillates. Urine analysis for metabolites although of limited scope and reliability is appropriate for monitoring compliance with treatment for the misuse of toluene and some chlorinated compounds.

Blood and Tissues

Headspace gas chromatography (GC) is well suited to the analysis of blood and tissues and has proved reliable in our hands for over 10 years. Recently, capillary columns have replaced the packed column to better address the resolution of now more common gaseous compounds (Figure 12) and reduce the analysis time (Streete *et al.* 1992).

Routine analysis may be reliably carried out using GC with a single capillary column (SPB-1, 60 m x 0.53 mm, 5 μ m) monitored with two detectors: a flame ionization detector (FID) and an electron capture detector (ECD). The effluent split ratio is set at 5:1 in favor of the FID. GC/mass spectrometry or GC/Fourier transform infrared spectroscopy may be required for

evidential work, but sensitivity can be a problem.

Great care needs to be taken to ensure that the sample is collected correctly to minimize loss of the very volatile compounds and also to ensure that it does not become contaminated with the suspected inhaled product during transport to the laboratory. In practice, for the majority of compounds, if the sample is taken by syringe, transported in a glass container of a suitable size (to minimize the headspace), and sealed with a cap lined with metal foil, then little loss will occur. The sample should be dispatched to the laboratory separately from any other case material, such as cans of glue, to avoid cross contamination. In the laboratory, the sample should be opened only when ready for analysis and only when cold (4°C) (Gill *et al.* 1988).

Accurate quantitative assay of the very volatile compounds (*e.g.*, butane) requires considerable care. The sample should be transferred to a preweighed headspace vial in which the assay is to be performed with the syringe and needle used to draw the specimen. It should be analyzed within 24 hours of collection (Figure 13).

Tissues are best analyzed after digestion in the headspace vial with an enzyme such as Subtilisin A (Novo Enzymes). A few milligrams of tissue can be dissected out of the center of a larger section of the frozen organ, then rapidly transferred to a preweighed headspace vial, capped, and treated with the enzyme solution and internal standard solution prior to incubation and analysis.

Trends in compounds causing death

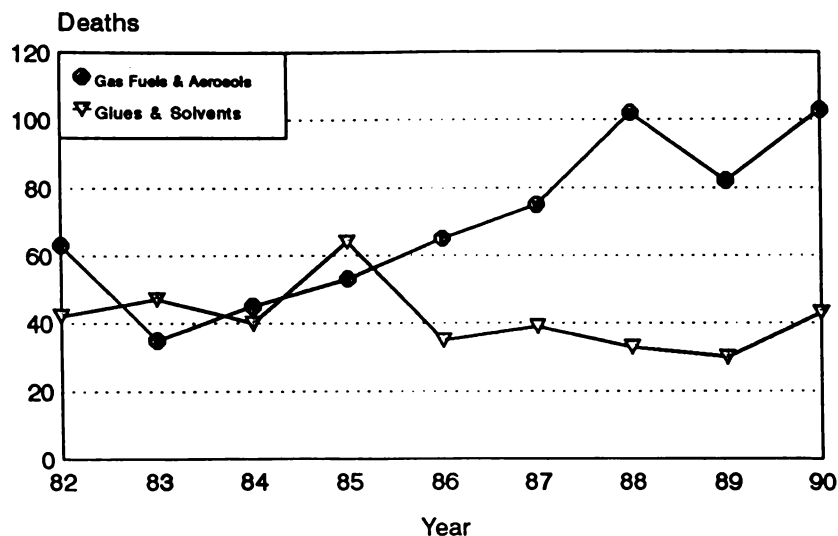


Figure 12.

Headspace analysis by GC

Blank

Add internal standard (200 μ L) to
headspace vial 7mL
Incubate 15 min at 65°C
Inject 150 μ L



Blank
Chromatogram

Sample

Add sample (200 μ L) to same vial
Re-incubate 15 min at 65°C
Inject 150 μ L



Sample
Chromatogram

Interpretation

Difference between Sample and Blank chromatograms

Calibration

Inject standard mixture (1 μ L) from
125mL gas sampling bulb



Standard
Chromatogram

Figure 13.

Breath

A transportable quadrupole mass spectrometer (PETRA VG gas analysis) with a membrane inlet was developed to support an epidemiological study of the neurological consequences of VSA (Chadwick *et al.* 1989). It was used to confirm that two populations, one VSA group and one matched control group were valid, and also that the abusers were not intoxicated when psychometric tests were administered. Seven mass fragments (in real time) were selected such that they would detect the presence of any of the compounds likely to be abused and the eighth channel used to monitor carbon dioxide to ensure a valid breath sample was obtained. The technique was validated against questionnaire and interview and proved effective and reliable even over extended use and when transported many miles by car. Two machines were also used during human volunteer exposure studies to investigate the kinetics of absorption of a range of volatile compounds (Gill *et al.* 1991).

A breath sample was then captured in the sampling system and a complete spectrum acquired for identification. A portion of breath (100 ml) was also passed through a solid phase trap for confirmatory analysis by GC at the laboratory.

Urine Analysis

Chlorinated compounds: Detection of trichloroacetic acid in urine by electron capture GC is a highly sensitive way of detecting exposure to trichloroethylene, and to a lesser extent, 1,1,1-trichloroethane and tetrachloroethane. We do not find trichloroacetic acid in the urine of unexposed individuals.

Toluene, Xylene: Hippuric acid results from the conjugation of benzoic acid with glycine. Benzoic acid is a metabolite of toluene, but is also added to food as an antioxidant, consequently detection alone is not proof of exposure to toluene. Hippuric acid is easily measured by reverse phase HPLC. It has been found empirically that, at least in the UK, a hippuric acid:creatinine ration (g/g) in urine in excess of one is highly indicative of toluene exposure. Xylene is metabolized in a similar way and the resulting toluric acids may be measured, they do not occur in the urine of exposed individuals.

Analysis of Products

It is important to maintain a healthy scepticism about the composition of domestic products, at least in the UK. Many products, particularly cleaning fluids,

change composition from batch to batch. Also during the transition to ozone benign propellants different batches of the same brand of aerosol product may contain different formulations, with examples of the older formulations remaining in the community for some time.

Vapor phase infrared spectroscopy is a quick and convenient way to examine the volatile components of most products suspected of abuse. A glass 10 cm path length cell with detachable sodium chloride windows (ca 4.5 cm diameter) and with 2 gas taps is convenient. The product, if gas or liquid, can be added by syringe or, if a solid (*e.g.*, glue), can be placed on aluminum foil out of the light beam. The spectra of mixtures of two or three components can usually be rapidly identified by reference to an in-house collection of reference vapor phase spectra.

Prevention

Education: The response to the problem in the 1970's was largely to use fear and revulsion very much like that used in early antismoking campaigns. By the 1980's, the response was to give harm reduction advice (*i.e.*, don't sniff alone or near water, don't sniff aerosols, be careful with plastic bags), often based on little or inaccurate information about the relative hazards of the different practices. More recently excellent educational packs have been produced for use in schools (TACADE). The problem is usually dealt with in schools under Personal and Social Education or Life and Social Skills where other drug issues are also dealt with. There is growing recognition that the majority of children with a VSA problem have other problems as well. Now the thrust seems to be to provide information to parents and at least alert them to the possibility that their children might be involved.

Considerable work has been done to educate retailers who have put in place voluntary restrictions on the availability of certain products, notably contact adhesives, to teenagers (Liss 1989).

Legislation

The only relevant legislation in England and Wales is the Intoxicating Substances (Supply) Act of 1985 which declares it an offense to sell products to persons under 18 years old in the knowledge or belief that it will be inhaled to achieve intoxication. Prosecutions

have been made under the Public Order Act of 1936, the Criminal Damage Act, and Section 5 of the Road Traffic Act. Actions have also been taken under bylaws controlling litter and the Railways Act controlling intoxication on railway property.

Awareness

The UK Department of Health has just completed a £ 1.4 m prime time television advertising campaign addressed at raising the awareness of parents that their children might be involved in VSA. The dramatic adverts encouraged parents to write or telephone for a free booklet on the subject, this was also made available from community pharmacies and doctors surgeries. Two million booklets have been distributed. The level of parental awareness was assessed precampaign and will be again shortly.

Labeling

There is much debate about product labeling, both whether this is desirable, or whether it just highlights products with abuse potential and what form of wording is accurate and acceptable to both the manufacturers and consumers. It is difficult to get across the message on a small label that a product is both safe in normal use and potentially lethal if misused in language that is comprehensible to an ill-educated 14-year-old.

Solvent-Free Products

Some manufacturers have produced substitute products that are solvent free. Solvent-free contact adhesives are available, but are not popular. A typewriter correction fluid is now available that is solvent (1,1,1-trichloroethane) free and works, unlike early attempts which were slow to dry. This approach has the added benefit that it also addresses the ozone depletion problem. However, it seems likely that there will always be volatile compounds in the community, it is difficult to see how the butane problem can be resolved.

Product Presentation

The two dominant products are gas cigarette lighter refills which are linked to 27.9% of all deaths and deodorants and antiperspirants which were involved in 9.5% of all deaths. One strategy would be to make the products less attractive (easy) to abuse or less hazardous.

Cigarette lighter refills are an unnecessary product when disposable cigarette lighters are cheap and readily available, but could be redesigned to make it more difficult to clench the nozzle between the teeth.

A modification to aerosol valves could make it impossible to get the propellant alone out by inverting the can and perhaps we will see compressed gas systems using air or nitrogen replace the existing technology.

Government Initiatives

Council of Europe: The Cooperation Group to Combat Drug Abuse and Illicit Trafficking in Drugs (Pompidou Group) has sent out a questionnaire to the Department of Health of member states asking about the level of awareness of the problem.

The British Home Office Advisory Council on the Misuse of Drugs has established a working party to address prevention strategies.

CONCLUSION

Anesthesia developed from observations made during the recreational use of volatile substances in the 1840s. Since then young inquisitive people have exploited the recreational possibilities of the substances made by the developing chemical industry and incorporated in domestic products. Now in the UK we see more deaths in teenagers from VSA than from all other forms of drug abuse. We have developed epidemiological and analytical (toxicological) tools to monitor the mortality, but have not adequately addressed the problem of systematically assessing the nature of the practice. Consequently, we are unable to advise about the relative risks posed by different substances, products, and practices. We observe a change to misuse of the more volatile compounds (gases) (Figure 12) and an increase in deaths (Figure 5), but are unsure if the two are related.

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Equine Drug Testing

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Drugs have been used to affect the performance of horses since recorded history. The Romans used elixirs of water and honey to increase the stamina of chariot horses and in the centuries that have followed, drugs have been used to improve performance. Doping to win as well as doping to lose ("nobbing") have been common occurrences (Mares 1958).

In the late 1800s in England and Europe, horse racing became a very popular sport. It was organized in each country under their respective Jockey Club. The purpose of the Jockey Club was to establish rules of racing and to conduct the sport of racing within these laws. With the introduction and understanding of the medical uses of opium in this era, drugging of horses became commonplace. Previous to this time, race horses were usually poisoned or drugged to lose because it was not generally recognized that horses could be stimulated to win. The use of opium and cocaine became so prevalent that they were given the name "dope" which was derived from the Dutch word "doop." This word referred to the stimulant potions used in tribal ceremonies by African natives. At the turn of the century, doping became such a problem that in 1903 the English and Irish Jockey Clubs created specific rules to prevent it. Their rule stated, "if any person shall administer, or cause to be administered, for the purpose of affecting the speed of a horse, drugs or stimulants internally, by hypodermic, or other methods, then he would receive the same penalty which faced all racings' wrongdoers, warning off." It is interesting to note that two American trainers, Enoch Wishard and John Keene, had taken horses to England to race as early as 1896 because gambling was generally not permitted in the United States. Needless to say, Wishard and Keene were reported to be the most skilled of the dopers and they made their mark on racing throughout the European continent.

Soon after the new rules concerning drugs were established, drug testing was initiated in Russia and

Europe. In 1903 the Moscow Jockey Club injected samples of race horse saliva into frogs and "warned off" American trainer, John Keene, for doping with morphine. In 1911 a Viennese chemist, Dr. Frankel, developed chemical tests to detect opiates and cocaine in horse saliva. Subsequently, chemical methods for drug testing were initiated in England and Europe which were associated with much legal challenge and controversy. By 1932 these tests which were basically color tests and microcrystal tests were implemented in the United States.

Drug testing has played an integral part in protecting and preserving the integrity of horse racing in the United States since the early 1930s. Unlike other sports and athletic competitions which have only recently developed an interest in the control of drugs, it has been a primary concern of horse racing for 60 years. During this time, racing commissions have spent millions of dollars to implement and improve drug testing procedures. Because of this commitment, racing has been successful in controlling the use of many illegal drugs which could affect the speed or racing performance of horses. Although it is impossible to detect all drugs which have abuse potential, it is possible for regulatory agencies to deter the use of many drugs through routine screening, targeted drug testing, and retesting frozen samples when new and improved analytical methods become available. Effective drug testing combined with significant penalties for violators remains the best deterrent to drug abuse in racing. It is probable that no amount of testing, research, or funding will ever place the testers ahead of the cheaters, but it is possible to create the situation whereby illegal drug use is not worth the fines and suspensions.

Equine drug testing is as great a challenge in the present day as it was at the turn of the century. There are three major reasons why the determination of drugs and their metabolites can be a challenge to the drug testing laboratory. First, a large number of drugs representing most drug classes must be detected.

Analytical methods are required that are very broad in scope and are capable of detecting many different drugs. Second, the amount of drugs and metabolites are often present in urine in low to subpart per billion concentration because very potent drugs are used. This precludes the use of many available methods which are not sufficiently sensitive and forensically defensible. Third, only limited funds are available to support this type of testing. Funds are often limited to as little as 10% of the cost per sample for testing in the International Olympics. In view of the pending fiscal cutbacks threatening racing at all levels, drug testing costs will become more significant in the future than ever before. Despite tremendous advances in analytical chemistry and associated technology, drug testing continues to struggle to keep pace with those who scheme of new ways to cheat and alter performance.

ILLEGAL DRUGS

In most countries of the world the intent of the rules outlined in the 1903 by the English Jockey Club to exclude drugs from racing still pertains. Stimulants, depressants, narcotics, local anesthetics, and any of their analogs which could affect the performance of horses are forbidden at any concentration. It is for this reason that most drugs are illegal and their presence is considered prima facie evidence that a drug has been administered in contravention to rules and regulations. Examples of illegal drug classes are shown in Table 1. A comprehensive list is beyond the scope of this paper. Exceptions include drugs such as phenylbutazone and furosemide which may be present in concentrations that have been established by regulatory agencies. The United States is the primary exception to this rule.

In the early 1960s phenylbutazone was introduced to horse racing in the United States. It was such an effective antiinflammatory and analgesic drug for musculoskeletal disease of the horse that it was hailed as a panacea and its use became permitted rather than forbidden (Gabel *et al.* 1977b). Horses which through injuries were hampered in racing without the drug were able to race competitively when treated with it. Its permissive use has been legalized to some degree in most racing jurisdictions. Similarly, furosemide (Lasix) was introduced in racing to treat a condition called exercise-induced pulmonary hemorrhage (EIPH) or bleeders (Gabel *et al.* 1977a). While the introduction of phenylbutazone stirred mild controversy, the permissive use of furosemide has created a heated debate that has continued to the present time (Tobin 1981). The primary reason for this is that furosemide has been demonstrated to enhance performance (Soma *et al.* 1985) and through its diuretic action, make drug detection of illegal substances much more difficult (Tobin *et al.* 1977). Despite scientific data which supports the logic to ban its use, furosemide has been classified as a legal drug by many racing jurisdictions in the United States.

DRUG DETECTION TIME

Due to the fact that any detectable concentration of drugs or their metabolites can be a violation, drug detection time is an important concept in equine drug testing. This is particularly important for drugs which have a legitimate therapeutic use to maintain the health of the horse prior to racing. Drug detection time is the interval during which the drug and/or its metabolites can be detected in either blood or urine following

Table 1. CLASSIFICATION OF DRUGS IN EQUINE DRUG TESTING ("ANY DRUG WHICH CAN AFFECT PERFORMANCE")	
Stimulants	
Depressants	
Narcotics	
Analgesics	
Others	Diuretics Steroids NSAID's Hormones Peptides, Proteins

administration. This period of time is also referred to as clearance time which is confusing. Clearance is a pharmacokinetic parameter which refers to elimination of the drug from the body and it is not necessarily the same as detection time.

Detection time depends on three factors: 1) the physico-chemical and pharmacological properties of the drug; 2) the dose of the drug; and 3) the sensitivity of the analytical method. The physico-chemical and pharmacologic properties of drugs govern their distribution in the body. Such is the case with an organic acid or an organic base and their associated dependency on "ion-trapping" within cells and across biological membranes. The dose of a drug can vary significantly depending on the desired pharmacologic effect and the resulting concentrations in blood or urine are dose dependent. The sensitivity of the analytical method, however, is the most important factor which determines detection time. Sensitivity of different analytical methods can vary over a several hundred-fold concentration range. The more sensitive the analytical method, the longer the detection time. Graphical representations of these concepts are shown in Figures 1 and 2. In Figure 1, note the markedly prolonged detection time associated with an analytical method which has been arbitrarily determined to be ten times more sensitive. In Figure 2, doubling the dose increases the amount excreted in urine but the detection time is

not prolonged compared to the use of a more sensitive method. For this reason, drugs must be administered to horses in different doses and urine samples must be tested by each method in the analytical protocol. These data serve as the basis for interpretation and enforcement of the drug rules.

SELECTION OF HORSES TO BE TESTED

The horses selected for testing should reflect the intent of the rules which is to prevent doping to win as well as doping to lose (Table 2). Ideally, the first- and second-place horses, the beaten favorite, and horses which do not perform according to form, should be tested. Approximately 30% of the starters are tested in this situation. Whenever fewer horses are tested, such as only winners, numerous drug classes, including depressants, could be excluded or eliminated by the selection process. A number of racing jurisdictions have reduced the number of samples to be tested as cost-savings measures. This selection process severely compromises the intent of the rules.

ANALYTICAL METHODS USED IN EQUINE DRUG TESTING

Equine drug testing is confronted with the same basic challenges in analytical chemistry that face forensic toxicologists. Two major differences associated with

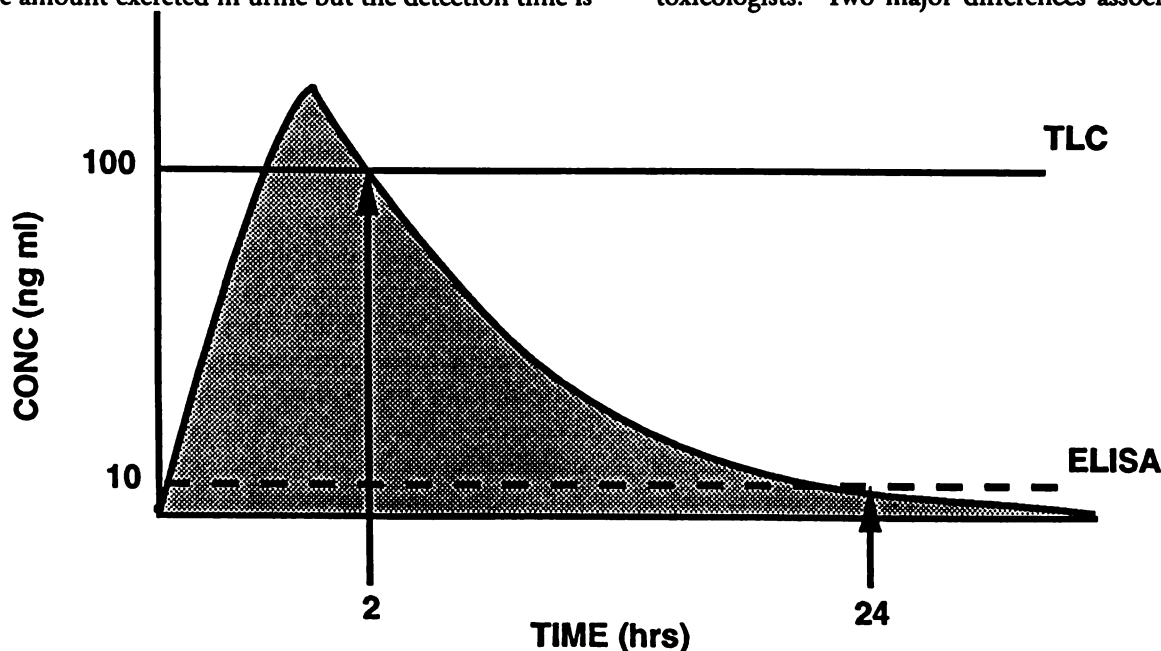


Figure 1. Detection time. Use of more sensitive analytical methods (ELISA) markedly increases the period of time that drugs can be detected relative to insensitive methods (TLC). The arrows indicate detection time. Sensitivity if ELISA has been arbitrarily set at 10 ng/ml whereas TLC has been set at 100 ng/ml. Time is on the abscissa and analyte concentration in ng/ml is on the ordinate.

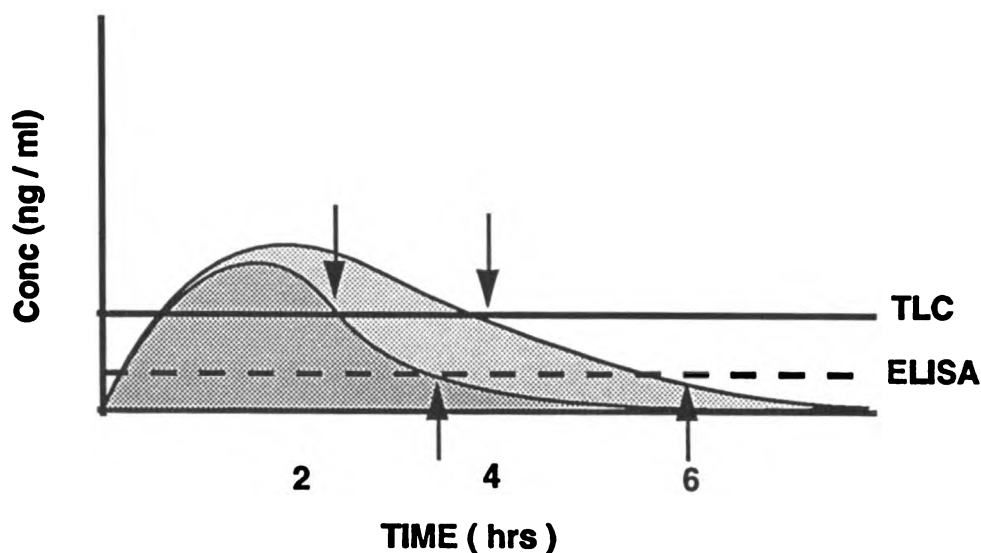


Figure 2. Detection time. Increased dose of drug has only a moderate influence on the period of time that drugs and metabolites can be detected. The arrows indicate the detection time for TLC and ELISA for two different drug doses. Detection time is on the abscissa and analyte concentration is on the ordinate.

Table 2. SELECTION OF HORSES TO BE TESTED IN EQUINE DRUG TESTING	
Horse Selection	
Winner	Always
Place (2nd)	In Combined Wagering
Show (3rd)	In Combined Wagering
Beaten Favorite	
Poor Performance	
30% of the horses should be tested.	

equine drug testing are the large number of drugs and the low concentrations of metabolites which must be detected. Many drugs are very effective or potent in horses. Drug doses as small as 0.0002 mg/kg body weight (0.1 mg per horse) are not uncommon. Consequently, only small amounts are required to achieve the desired, subtle, pharmacological response. This in turn accounts for the low urine concentrations of analytes. Further complicating the problem is the very complex biological matrix which gives rise to high background interferences with most analytical methods.

Most analytical methods available for use in routine laboratory work have been evaluated and used in equine drug testing. The following methods have been used with varying degrees of success: microcrystal test, color or spot tests, thin layer chromatography

(TLC), gas chromatography (GC) equipped with flame ionization detectors, electron capture detectors and nitrogen-phosphorus detectors, high-performance liquid chromatography (HPLC) equipped with ultraviolet, photo diode array spectroscopy, fluorescence and electrochemical detectors, capillary electrophoresis, infrared spectroscopy, nuclear magnetic resonance spectroscopy, gas chromatography/mass spectrometry (GC/MS) in the full scan electron impact (EI) and selected ion monitoring (SIM) modes, liquid chromatography/ mass spectrometry (LC/MS) with various soft-ionization techniques, and mass spectrometry/mass spectrometry. Similarly, all available extraction and purification techniques have been evaluated including liquid-liquid, solid phase, and supercritical fluid. Last but not least, immunoassays (IAs) have been developed and used extensively for

general screening and targeted assays. The methods which have been most commonly used in equine drug testing and the reasons for their selection are outlined below.

The general approach used by laboratories involved in equine drug testing involves three separate steps. The first step is general screening, the second step is targeted drug detection, and the third step is drug confirmation. The complex matrix of horse urine combined with the requirement of analyte detection in the low to subpart per billion limits the utility of most analytical methods. No single analytical method is suitable to detect all drugs and/or their metabolites in horse urine. There is no best analytical method or required criteria upon which equine drug testing methods are based. A combination of detection methods are generally used in order to generate enough consistent analytical data to withstand forensic challenge. Each equine drug testing laboratory uses methods developed primarily within their own facility. A national drug testing and quality assurance program is under development, but it has not yet met its mandate.

DRUG SCREENING

Drug screening is the most difficult aspect of equine drug testing because seldom is there any associated information or clue to indicate what specific drug or class of drugs may be present. A race horse which has been successfully medicated illegally shows no outward clinical signs of tampering. The analytical challenge, therefore, is to determine which samples may contain an illegal drug and which samples are negative.

The ideal drug screening procedure should be sensitive, inexpensive, rugged, dependable, easy to use, and capable of detecting many drugs. Subpart per billion concentrations of drug metabolites must be detected in many instances. The total available fee for testing an equine sample ranges between \$20 - \$50. Two basic approaches which are widely used include chromatographic procedures and IAs.

The approach that has been used most effectively employs various chromatographic methods including TLC, GC, or HPLC (Dewey *et al.* 1981). These techniques are widely used and well established. They are limited, however, by the need for extensive extraction and cleanup procedures which are laborious and time consuming for the routine testing laboratory.

TLC is the least expensive, most versatile, and most commonly used of the available chromatographic methods in equine drug testing. On an absolute basis, it is not as sensitive as HPLC or GC but its sensitivity is comparable and often better when biological extracts are assayed. This is especially true for drugs or metabolites which are amphoteric, polar, thermally unstable, and lacking in strong detector response. Sympathomimetic, beta-agonist drugs shown in Figure 3 represent this class of compound. Concentrations of metabolites as low as 50 ng/ml urine can be detected by TLC. In many instances TLC is three to five times as sensitive as GC/MS or LC/MS in the full scan mode. In addition, use of TLC scrapes for off-line, sample cleanup is often required before either of these techniques can be successfully employed. In a comprehensive TLC drug screening program, as many as 9 different extracts are used in conjunction with up to 16 different TLC plates sprayed with a variety of detection reagents. In such a scheme, following conjugate hydrolysis, acid, base, neutral, amphoteric metabolites can be readily detected at concentrations greater than 50 ng/ml urine. In side-by-side comparisons with other techniques, TLC has demonstrated its utility, importance, and critical role in equine drug testing. It can readily detect the metabolites of promazine (Figure 4) at concentrations of approximately 20 ng/ml whereas GC and HPLC are less sensitive.

GC and HPLC screening methods have not gained wide use in equine drug testing as it has in other forms of sports drug testing (Catlin *et al.* 1987; Park *et al.* 1990a; 1990b). The primary reason is due to the limited scope of drugs and metabolites which can be detected. In addition, GC and HPLC become cost-prohibitive in the event that a battery of tests is conducted to detect different drug classes. In general, all chromatographic methods have marginal sensitivity when used in the context of general drug screening for potent drugs due to the interference of background constituents.

Sample preparation or purification is the most important step involved in the successful application of chromatographic procedures in trace drug determination in a biological matrix. Its importance cannot be overemphasized. It is also the most ignored. Multiple liquid-liquid extractions which utilize solvents of varying polarity and back-washes are required for drugs with different polarity and function groups. Horse urine severely limits the usefulness of solid phase extractions because of its high ionic strength and mucoid nature.

Crude horse urine plugs columns and markedly impairs extraction efficiency. The combined use of liquid-liquid and solid phase extraction provides the best recoveries and the cleanest extracts. Time spent on sample cleanup, the "front end" of the analysis, prolongs column life, reduces detector fouling, and invariably gives better detector response and spectra.

The second approach utilizes IAs. IAs are advantageous and will be discussed in some detail because sample preparation is either not required or minimal and they are the most sensitive methods available for drug screening. Detection in picogram/ml concentrations is possible. Radioimmunoassay, enzyme multiplied IA tests, fluorescence polarization immunoassay, particle concentration immunoassay, and enzyme-linked immunosorbant assay (ELISA) tests have been available for a limited number of drugs. The use of IAs have been limited due to cost and the small selection of test kits which have been commercially available.

IA tests are often not analyte or drug specific. They cannot provide unequivocal identification compared to conventional confirmatory methods involving chromatography and spectroscopy. An antibody developed to a hapten usually recognizes or "cross-reacts" to some extent with closely related compounds or analogs. Depending on the specificity of the antibody produced, an IA may be highly drug specific, analog specific, or class specific. A drug hapten for morphine is shown in Figure 5 and the resultant cross-reactivity for opiates is shown in Figure 6 and Table 3. It is difficult to predict the specificity of an IA in either polyclonal or monoclonal systems. Each antibody must be evaluated individually within the scope of the delivery system. Monoclonal systems may not be more specific than polyclonal antibody (McKay *et al.* 1990). IAs with a high degree of specificity are also available including the detection of specific optical isomers and specific drug metabolites (Fitzgerald *et al.* 1988).

The nonspecificity of IAs can be an advantage as well as a disadvantage. In general screening, nonspecificity serves the useful purpose of excluding negative samples and indicating or flagging samples which may contain an illegal drug. In an IA test, a positive finding only indicates that one of at least several different drugs could be present. Conventional confirmatory methods (GC/MS) are required to determine the chemical identity of the substance

producing the immunoreactivity. This is relatively simplistic compared to the problem of determining what sample contains a drug. Use of IA to estimate concentrations of the immunoreactive substance in unknown samples is not possible. The presence of metabolites which have varying immunoreactivity, reactive analogs, and nonspecific binding can produce very misleading results. For this reason, IAs are best used as preliminary screening methods with defined cutoff values (Baselt 1989).

New IAs specifically designed to detect a large number of drugs of importance in equine drug testing are now commercially available by several vendors. Their availability clearly makes IA testing the method of choice in drug screening and targeted drug determination.

The Cornell IA (CIA) tests represent the new class of IAs which have been specifically designed for drug screening in equine testing. They are manufactured and validated in the author's laboratory. CIA tests are two-step ELISA. Synthetic antigens are coated on the polystyrene microtiter plate wells rather than the antibody as done in one-step tests. In the first step, drug specific antibodies are added to urine as competitive binding reagents. In the absence of targeted drugs, the antibody binds to the bound antigen in the polystyrene microtiter well. In the presence of drugs, a drug-antibody complex is formed which cannot react with the bound antigen and it is removed from the well in a wash procedure. In the second step, species-specific antibody or second antibody which has been labeled with horseradish peroxidase enzyme is added to the wells. The species specific antibody binds to any drug antibodies attached to the bound antigen. Upon the addition of substrate (TMB), color development occurs which is in direct proportion to the amount of second antibody bound to the microtiter well. The amount of second antibody available for binding is in indirect proportion to the amount of targeted drug or analyte present in the first step. A negative urine produces a strong blue color in the test whereas a positive sample has no color development.

The two-step ELISA technology offers several advantages over other ELISA tests. First, the precious first antibody is added to the wells in only small amounts. The synthetic antigen which is easily prepared is coated on the wells and the antibody is not wasted. Secondly, the additional wash step serves to eliminate interfering background material. Thirdly, the concentration of

immunoreactants can be readily optimized for maximum sensitivity and stability. Finally and perhaps most importantly, this technique permits the production of test kits for as little as \$.25 per well.

At the present time, 44 CIA tests have been developed. The list is shown in Table 4. Some tests are highly compound-specific whereas others are class-specific. Many more assays are in various stages of development. The objective of our drug-testing program is to develop at least 75 IAs, of which at least 30 tests would be used, per sample, in drug screening. The associated cost for the IA part of the test would be less than \$10 which is an affordable price. Once a large battery of IAs is in place, dependency on chromatographic screening methods will be reduced to the status of targeted detection for compounds which are not detected by IAs.

TARGETED DRUG DETECTION

Targeted drug detection is a limited form of drug screening. The intent is to detect a limited number of predetermined compounds with a specific analytical test. By limiting the scope of drug coverage, greater sensitivity is usually achieved and greater specificity is obtained. IA or chromatographic methods are used effectively in this context. Targeted testing is seldom used in equine drug testing because there is not one group of drugs which requires more attention or better detection than another.

DRUG CONFIRMATION

Drug confirmation is the basis upon which results stand or fall. The "gold standard" for drug confirmation in equine drug testing is capillary gas chromatography combined with full scan, electron impact, mass spectrometry. In the absence of full scan EI data, SIM combined with at least one additional combined chromatographic-spectroscopic technique is desired such as HPLC photodiode array spectroscopy. As stated above, detection of drugs and metabolites in the full scan mode at concentrations below 10 ng/ml are difficult but achievable through judicious use of sample cleanup procedures and derivative formation. TLC scrapes are the most commonly used method to purify samples for low-level confirmation. Chemical ionization GC/MS in positive or negative ion modes is equally problematic in the full scan mode. Necessary data can usually be

achieved, however, through extensive sample cleanup. For example, the lower limit of detection in the full scan mode with these conventional techniques for benzoylecgonine in horse urine is approximately 1 ng/ml.

LC/MS techniques which have the necessary absolute sensitivity requirements for pure analytical standards are severely hampered by the high background interference of equine urine. This is indeed the case for LC/MS techniques which require microbore columns and low flow rates. These technique are not rugged enough for forensic work. Consequently, LC/MS is not used routinely in equine drug testing.

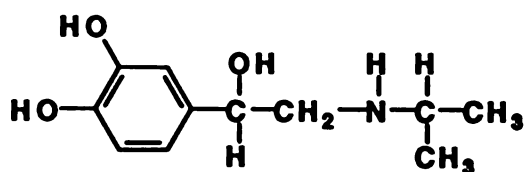
SUMMARY

Detection of drugs and metabolites in horse urine is a difficult challenge. The large number of drugs which must be detected at low to subnanogram per ml concentrations requires optimal performance from available analytical methods and instrumentation. Highly effective, IA methods have been developed which are ultrasensitive and inexpensive for use in drug screening. Confirmatory techniques involving chromatography and spectroscopy are problematic. The absolute sensitivity which can be achieved with pure standards can seldom be matched with equivalent concentrations in biological extracts. Extensive sample preparation and cleanup are, therefore, required in order to confirm the identity of the analytes flagged by IA.

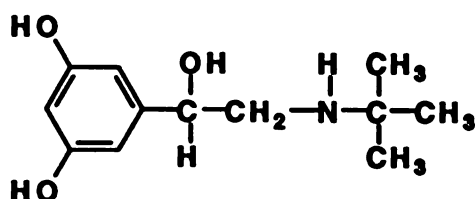
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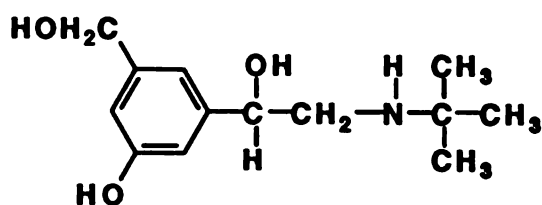
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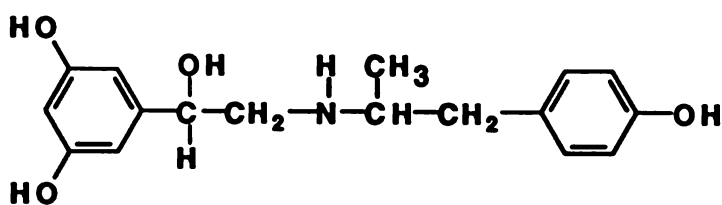
ISOPROTERENOL



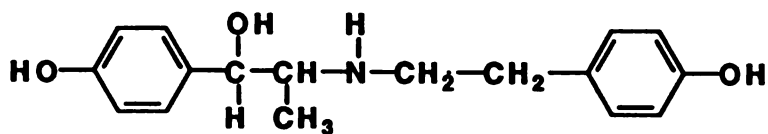
TERBUTALINE



ALBUTEROL



FENOTEROL



RITODRINE

Figure 3. Structures of sympatomimetic beta-agonist drugs. These compounds represent polar, amphoteric, thermally unstable compounds which are difficult to extract and difficult to detect using conventional chromatography.

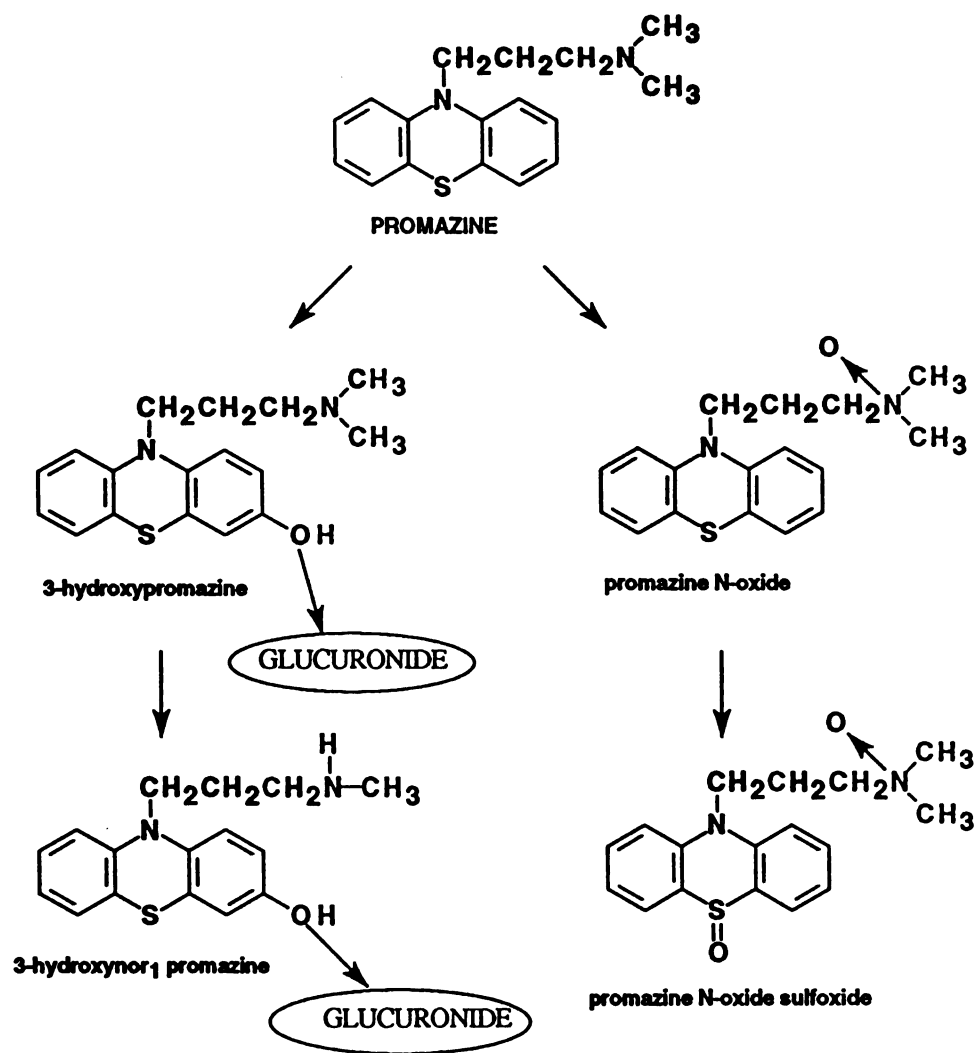
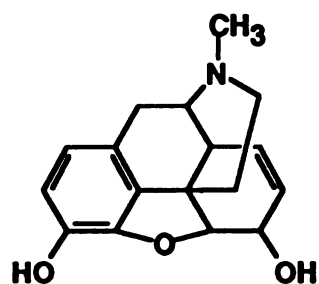
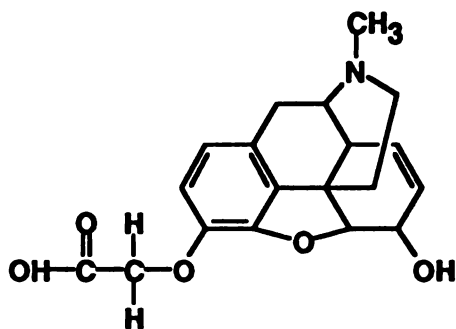


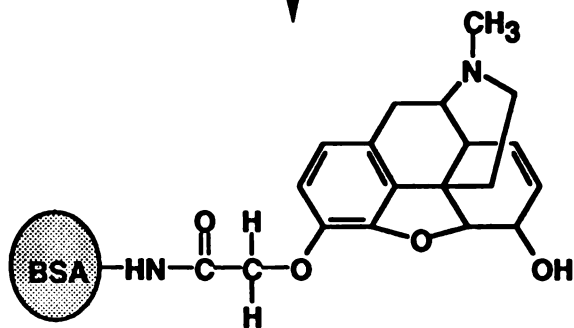
Figure 4. Structures of promazine and its metabolites. These compounds are readily detected by TLC whereas they are problematic to detect by means of HPLC and GC.



MORPHINE

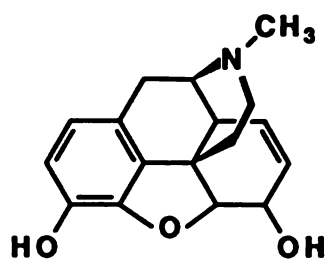


3-O-CARBOXYMETHYLMORPHINE

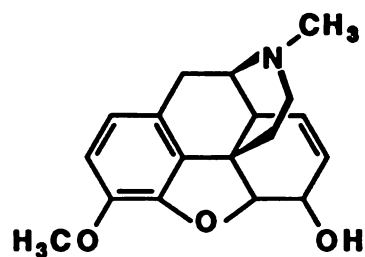


IMMUNOGEN

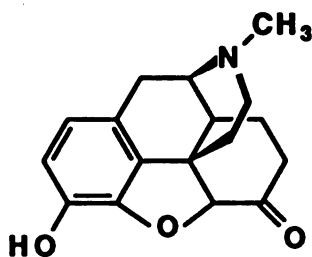
Figure 5. Structures of the hapten and immunogen used to develop antibodies to morphine and opiate analogs.



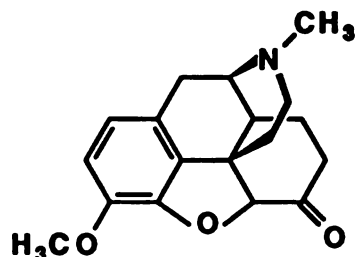
MORPHINE



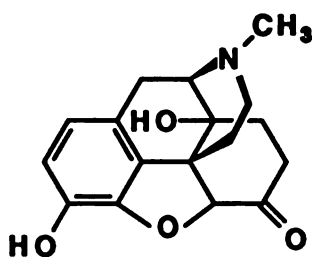
CODEINE



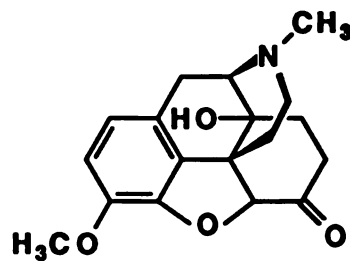
HYDROMORPHONE



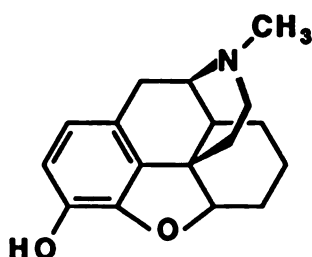
HYDROCODONE



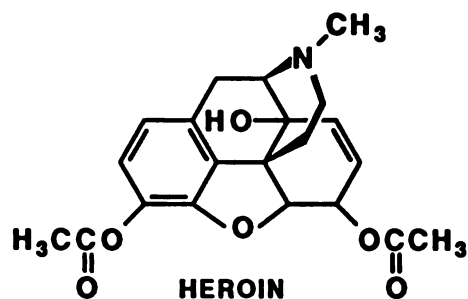
OXYMORPHONE



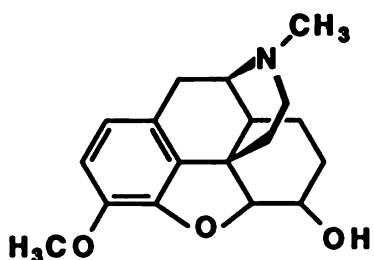
OXYCODONE



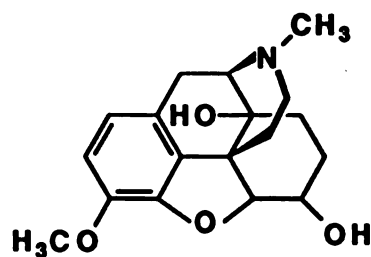
LEVORPHANOL



HEROIN



DIHYDROCODEINE



DROCODE

Figure 6. Structures of opiate drugs which are immunoreactive to polyclonal antibodies developed to 3-O-carboxymethylmorphine.

Table 4. LIST OF CORNELL IMMUNOASSAY TESTS FOR EQUINE DRUG TESTING

ELISA FORMAT	IMMUNOREACTIVITY		ELISA FORMAT	IMMUNOREACTIVITY
Acepromazine (Primary)	Acepromazine, Promazine Chlorpromazine Propionylpromazine Trifluorpromazine Amitriptyline Imipramine Chlorprothixene		Haloperidol	Haloperidol
Acepromazine (Secondary)	Propiomazine Trifluorpromazine Methotrimeprazine Promethazine		Ipratropium	Ipratropium
Acetophenazine	Piperacetazine Carfenazine, Pipothiazine Flupenthixol Acetophenazine		Ketorolc	Ketorolac
Albuterol	Clenbuterol, Terbutaline Pirbuterol, Nisbuterol Carbuterol, Bitolterol Sulfonterol, Colterol Albuterol		Methadone	Methadone
Alfentanil	Alfentanil		Methamphetamine	Methamphetamine, MDMA
Anileridine	Anileridine, Meperidine		Methylprednisolone	Methylprednisolone Prednisone, Prednisolone
Atropine	Atropine		Nalorphine	Nalorphine, Levallorphan
Azaperone	Azaperone		Opiates	Morphine, Codeine, Heroin Hydromorphone, Metopon Hydrocodone Oxymorphone, Oxycodone Dihydrocodeine Levorphanol, Thebacon
Barbiturates	Most Barbiturates		PCP	Phencyclidine
Benzodiazepines	Those Which Metabolize to Oxazepam		Pentoxifylline	Pentoxifylline
Buprenorphine	Buprenorphine		Pehnm trazine	Phenmetrazine Phendimetrazine
Butacaine	Butacaine		Prednisolone	Prednisolone, Prednisone
Butaperazine	Butaperazine Prochlorperazine Trifluoperazine		Procaine	Procaine
Butorphanol	Butorphanol, Nalbuphine Pentazocine		Propiomazine	Propiomazine Promethazine
Carfentanil	Carfentanil		Reserpine	Reserpine (Plasma)
Cocaine	Benzoylcegonine		Spiperone	Spiperone
Detomidine	Carboxydetomidine		Sufentanil	Sufentanil
Dexamethasone	Dexamethasone Betamethasone		THC	Theophylline
Etorphine	Etorphine		Triamcinolone	Triamcinolone
Fentanyl	Despropionylfentanyl Carboxyfentanyl		Tricyclics	Amitriptyline Nortriptyline, Imipramine Desipramine
Flunixin	Flunixin		Zomepirac	Zomepirac
Fluphenazine	Fluphenazine Perphenazine			
Furosemide	Furosemide			

Hair Analysis for Drugs of Abuse: Forensic Issues

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Hair has long been recognized as a suitable specimen for forensic and toxicological investigations. During the early 1970s, interest focused on toxic metal analysis (lead, mercury, and arsenic). Subsequently, the focus shifted to the analysis of biomedically essential trace metals (selenium, chromium, iron, etc.) and, in recent years, to drugs of abuse (Baumgartner *et al.* 1979).

The biomedical trace element field, however, was soon to be embroiled in controversy. This came about mainly because of the difficulty of establishing a link between health problems and trace metal content of hair. Of course, a link with health status is more apparent for toxic metals and for drugs of abuse.

One encounters several technical problems when hair analysis is performed with human subjects and for analytes such as drugs of abuse. With animals, for example, analysis of hair is facilitated by a favorable hair-to-body-weight ratio. Unfortunately, this is not the case when human subjects are involved. Consequently, large quantities of human hair (several grams) are required for the analysis of heavy metals by even such sensitive techniques as atomic absorption spectroscopy. Individuals rarely object to providing such large quantities of hair when a health problem is being evaluated. However, when testing for drugs of abuse, a sample of only approximately 1/100 of the amount used for metal analysis can be obtained. Furthermore, because of the indestructible nature of metals, all metal analytes can be readily released by the same convenient extraction procedure—perchloric acid destruction of the protein matrix. In the case of drug testing, convenient extraction is precluded by the different chemical stabilities and solubilities of the analytes. Most important, however, hair analysis for drugs of abuse has to meet or exceed the strict criteria of forensic proof developed for urine

drug testing. The present paper focuses extensively on this aspect of hair testing.

PASSIVE DRUG EXPOSURE: THE EVIDENTIARY FALSE POSITIVE PROBLEM

It is one thing to identify and measure drugs of abuse or their metabolites in biological specimens such as urine, blood, hair, perspiration, or saliva and it is quite another matter to accurately interpret such findings as being caused by active drug use as distinct from passive exposure. Passive exposure may occur by the ingestion of poppy seeds, spiked food or drink, the internalization of environmental contaminants, or (in the case of hair) by external contamination of the specimen. Examples of individuals who are typically and chronically exposed to environmental drug contaminants are the spouses of drug users, narcotics agents, and drug dealers.

In the case of body fluid analysis, such environmental contaminants, when internalized through the lungs or gastrointestinal tract, give rise to positive results which cannot be distinguished from active use. We designate such results as evidentiary false positives to distinguish them from technical false positives, such as the misidentification of a substance by mass spectrometry.

Unlike hair, the gastrointestinal tract or lungs offer no resistance to environmental contamination. As a matter of fact, the active inhalation of vapor is unfortunately a very effective means of internalizing environmental drug contaminants, which in the case of cigarette smoke actually manifests itself as an elevated cancer risk. In contrast to this, we all know that the smell of cigarette smoke can be readily removed from hair by shampooing. Although the body's interior milieu is constantly being cleansed by urinary excretion, it is

Table 1. CUTOFF VALUES USED IN DRUG TESTING				
Substance	Urine (ng/ml)		Hair (ng/ml)	
	Immunoassay	GC/MS	Immunoassay	GC/MS
Marijuana Δ9-Carboxy-THC	100	15	5	0.1
Cocaine Equivalents Benzoylecgonine	300	150	500	500
Opiates Morphine Codeine	300	300 300	500	300 300
Phencyclidine (PCP)	25	25	300	300
Amphetamines Amphetamine Methamphetamine	1000	500 500	500	300 300

evident that these processes are not sufficient to guard against evidentiary false positives in cases of chronic passive drug exposure, such as that experienced by the spouse of a heavy drug user. A number of studies have shown that quite small quantities of drugs can generate such evidentiary false positives for urinalysis (Baselt and Chang 1987; Baselt *et al.* 1991).

Appropriately set cutoff levels are virtually the only means available to urinalysis for distinguishing between active drug use and passive exposure. However, very little research appears to have gone into the determination of these cutoff values. In the case of marijuana, many argue that the cutoff level of 100 ng/ml for the screening test is too high and that it was set mainly to meet military policy needs. In contrast to this, the opiate cutoff level of 300 ng/ml is considered too low to guard against evidentiary false positives from poppy seed ingestion.

As the following will show, hair analysis can assist urinalysis in resolving its evidentiary false positive problems. But first let us look at how this problem can be handled in the case of hair analysis.

Preventing Evidentiary False Positives in Hair Analysis

Hair analysis offers many more approaches than urinalysis for avoiding evidentiary false positives. Also,

the passive exposure problem of hair can be more readily investigated than that of urine. For example, the most significant issue—external contamination—can be studied exhaustively with isolated hair specimen (*i.e.*, without the problematic participation of human subjects). The scientific basis for the criteria used for preventing evidentiary false positives is, therefore, more readily established for hair than for urine.

Two types of passive drug exposure must be considered in the case of hair analysis: (1) endogenous exposure, or the entry of drugs into hair from the bloodstream as a result of the internalization of environmental drug contaminants; and (2) exogenous exposure, or the direct deposition onto or into the hair fiber of drugs present in the environment.

Endogenous Passive Drug Exposure

Endogenous contamination, which is so troublesome for urinalysis, can for all practical purposes be ignored in hair analysis. The reason for this is that drugs become entrapped in hair at levels which are approximately correlated to the quantity of drugs taken in. Consequently, the relatively small amounts of drugs which are absorbed by passive exposure, and which do not generate psychotropic effects at these levels, accumulate in hair in quantities which are well below the cutoff levels used for hair analysis (Table 1). This situation arises because the cutoff levels for hair are not

based on physiological criteria, as they are with urinalysis, but instead are based on analytical criteria [*i.e.*, on the maximum attainable sensitivity of either the screening assay or the GC/MS/MS (gas chromatography/tandem mass spectrometry) confirmation procedure]. As it turns out, the cutoff levels for 3 of the 5 drugs in Table 1 are actually higher with hair analysis than with urinalysis.

Further protection against false positives due to passive endogenous drug exposure is provided by the ratio of the highest reported drug levels in specimens to the values of their cutoff levels. The lower the ratio, the greater the protection. In the case of hair this ratio is in the range of 100 and in the case of urine it is around 1,000. Marijuana is an exception in this respect.

Hair analysis advantages relative to urinalysis with respect to endogenous passive exposure was demonstrated in a study in which poppy seeds were ingested by several volunteers on a daily basis for 1 month in quantities to yield positive urine values in the range of 500 ng/ml. At these levels, opiates were not detectable in hair.

Exogenous Passive Drug Exposure

An example of exogenous exposure would be the contamination of the hair specimen through contact with drug vapors or powders. Our experience with specially developed procedures, which have been applied to over 150,000 hair samples, has demonstrated that evidentiary false positives due to external contamination can be avoided with a high degree of certainty.

These procedures were developed through an extensive series of investigations which demonstrated the following important properties of hair:

1. High resistance to contamination by externally applied drugs;
2. Ready removal of externally applied drugs from hair by various wash procedures;
3. The inability to remove drugs entrapped in hair from the bloodstream by washing;
4. Unique wash/extraction profiles distinguishing between exogenous and endogenously derived drugs; and

5. Identification of entrapped metabolites in hair.

Contamination and Decontamination Studies

It is generally known from research in the cosmetic industry that hair is highly resistant to contamination by externally applied organic substances and that any adhering substances (*e.g.*, cigarette smoke, dirt) can be readily removed by conventional shampoos (Robbins 1988). We initiated extensive studies to establish whether the principles of surface chemistry established in the cosmetic industry for the deposition and removal of organic substances from hair applied to the presently investigated drugs of abuse. As these studies were described in detail elsewhere (Baumgartner and Hill 1992; 1993), they will be only briefly reviewed in the present report. Specifically, the questions we wanted to answer were:

1. To what extent can hair be contaminated by externally applied drugs in solution or in the vapor phase?
2. To what extent is contamination influenced by the structural integrity of the hair?
3. How strongly bound are the exogenously derived drugs (*i.e.*, how readily can these be removed from damaged and undamaged hair by various cleaning agents)?
4. How do the above properties for exogenous drugs differ from those for endogenous drugs?

The resistance of hair to contamination by drugs of abuse was evaluated under exposure conditions which far exceeded anything that would likely occur in everyday situations. We adopted these extreme conditions rather than more realistic ones because of our interest in forcing measurable quantities of drugs into the interior structures of the hair from which their removal could be followed by wash kinetic experiments. Their rate of removal was then compared to that of endogenously deposited drugs. For example, in the case of cocaine we placed hair into a 1-liter container in which .5 gm of freebase cocaine was vaporized for several hours. In the case of aqueous cocaine exposure (an extreme model for contamination by sweat), hair was soaked for 3.5 hours in solutions (5 ug/ml) of cocaine hydrochloride. Our experiments were therefore performed under conditions which were more appropriate for preparation

of controls than for evaluating contamination.

The results in Figure 1 show that hair is highly resistant to penetration by cocaine vapors. This can be seen from the large quantities of cocaine adhering loosely to the hair surface relative to the small amount of material requiring more extensive washing for removal. The rapid attainment of a plateau in the wash kinetics and the insignificant amounts of drugs found upon dissolving the hair attest to the effectiveness of the wash procedures. Interestingly, the effectiveness of the wash procedures are identical for detergent and nondetergent-based procedures. Also, no difference was observed between washes with hair-swelling and nonhair-swelling solvents (*i.e.*, water and ethanol, respectively).

Hair of different porosities (*i.e.*, with increased cuticle damage as evidenced by uptake of methylene blue stain), when exposed to cocaine hydrochloride solution, took up drugs in proportion to their porosities (Figure 2). The relatively intact hair (Specimens B & C) exhibited a high degree of resistance to penetration by cocaine. Only with the highly porous hair (Specimen

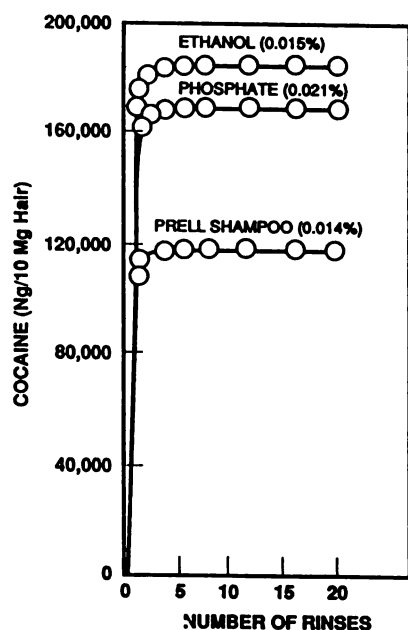


Figure 1. Cleaning of hair with multiple washes (1 - 2 minutes) at 37°C subsequent to contamination of hair specimens with cocaine vapors. Washes were performed with different solvents: Prell shampoo, phosphate buffer (pH 6), and ethanol. Residual amount of cocaine in hair after washing is expressed as a percentage of the total removed.

A) was there significant uptake of cocaine hydrochloride in domains less accessible to the wash solutions. This is reflected by the gently sloping second phase of the wash kinetic curve instead of the rapid attainment of a plateau. Most important, however, no significant quantities of residual cocaine hydrochloride contaminant were found at the conclusion of the wash procedures in the three different types of hair specimens.

The results obtained with the other drugs of abuse—opiates, methamphetamine, phenylcyclidine, and marijuana—were essentially identical to those obtained with cocaine (Baumgartner and Hill 1992; 1993). Our studies indicate, therefore, that hair is highly resistant to the penetration of exogenous drugs and that they bind only weakly to hair, thereby facilitating their ready removal by conventional wash procedures.

Several investigators have advanced the opinion that exogenous drugs of abuse bind firmly to hair by processes analogous to that occurring with hair dyes (Kidwell and Blank 1992). This is a highly misleading position, for not only is it inconsistent with our

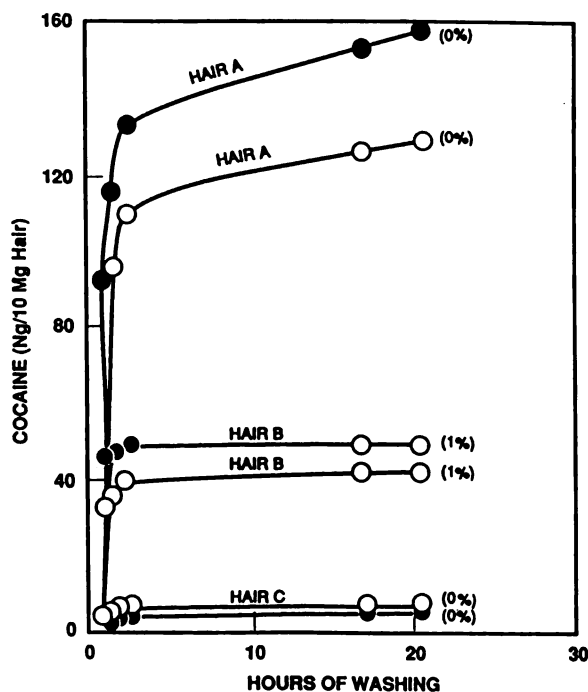


Figure 2. Cleaning of different types of hair with different types of solvents subsequent to contamination of hair by soaking specimens in cocaine hydrochloride (5 ug/ml) for 3.5 hours at 37°C. Types of hair: A, porous treated hair; B, adult untreated hair; C, juvenile untreated hair. Solvents used: ●, phosphate buffer, pH 6; ○, ethanol. Residual amount of cocaine in hair after washing is expressed as a percentage of the total removed.

contamination studies, but also with virtually everything that is known about the technology of hair dyeing (Robbins 1988). For example, there are essentially only four classes of dyes: permanent or oxidation dyes, semipermanent dyes, color rinses, and metallic dyes. Permanent dyes generally consist of p-diamine and p-aminophenols which are oxidized by hydrogen peroxide to active intermediates. These then react in hair with color couplers to provide shampoo-resistant dyes. Obviously, such reactions are not involved with drugs of abuse. Semipermanent dyes diffuse into the hair, but are not firmly bound as evidenced by their removal by four to six washes with shampoo. Temporary dyes are acid dyes which do not bind firmly and may be removed by a single shampooing. Metallic dyes act in a manner similar to trace metals.

It is also inappropriate to compare the contamination problems of trace metals in hair with contamination by drugs of abuse. For one, trace metals are far smaller than drug molecules and consequently enter the protein structure of hair more readily. Furthermore, metals generally form very strong covalent or ionic bonds with protein functional groups, in particular with SH groups. And, of course, metals are ubiquitously present in the environment, particularly in cosmetic agents and in the water supply.

Differentiation Between Endogenous and Exogenous Drugs

We carried out extensive studies to establish whether hair containing endogenously incorporated drugs due to drug use presents a different wash kinetic profile than hair containing only exogenously deposited drugs. If a difference were to be found (*e.g.*, the inability to remove endogenous drugs from inaccessible domains in the hair), then such a difference would constitute a method for differentiating between drug use and environmental contamination.

We concur with other investigators that the measurement of metabolites is another effective means for making this distinction. But it is always desirable to have, whenever possible, several independent methods for forensic assessments. And then, of course, there are also drugs which either do not produce metabolites or where metabolites are present in concentrations that can only be measured by ultrasensitive means (*e.g.*, GC/MS/MS). In addition, wash procedures guard against subversive activities such

as willful contamination of a hair specimen with metabolites. And finally, in certain circumstances it is preferable to measure drug/metabolite ratios rather than the absolute levels of metabolites (*e.g.*, cocaine/benzoylecgonine, methamphetamine/amphetamine). For accurate ratio measurements it is important to remove all exogenous drugs. If this is not done, drug users could change the interpretation of the test results to one of contaminated nonuser merely by the willful contamination of the hair with exogenous drugs.

Therefore, for all of the above reasons it is important to pay attention to the theory and practice of hair washing. As will be shown below, this requires a detailed kinetic analysis of drug removal relative to the amount of drug remaining in hair.

Wash Procedures

We have adopted two types of wash procedures: (1) the extended wash procedure; and (2) the truncated wash procedure. In the extended procedure we performed washes until the wash kinetics showed a plateau. The duration and number of washes were defined by the results obtained with a particular sample. This procedure involved, therefore, the analysis of wash solutions while the wash procedure was being performed. In the truncated procedure, the number of washes and their duration were fixed, and the results were related mathematically to an extended procedure by three wash kinetic criteria.

The truncated wash procedure was used for mass production testing, whereas the extended wash procedure was used for the investigation of forensic cases, or for samples which fall outside our wash kinetic criteria by the truncated procedure, or samples where the results of the first hair analysis with the truncated procedure are challenged ("safety net" samples).

Before commencing the washing, it was desirable to establish the porosity of the hair by methylene blue staining (0.5% methylene blue, 5-minutes contact time followed by copious rinsing, and microscopic examination). Intact (nonporous) hair exhibits no uptake of dye whereas stain is readily taken up by porous hair.

There are several causes for increased porosity of hair; the most frequent are cosmetic treatments such as perming, dyeing, and relaxing. In such cases it is

important to separate the treated from the untreated part of the hair. Other causes of increased permeability that we have encountered involved cases where the hair was maintained in an unfavorable environment (*e.g.*, the remains of a murder victim 2 years after the murder) or exceptionally old hair (*e.g.*, a 165-year-old hair specimen from the poet John Keats and 600-year-old hair from a Peruvian mummy). Drug residues were identified in all of these specimens.

An appropriate wash solvent was then chosen based on the porosity of the hair. Nonporous to slightly porous hair was washed with phosphate buffer (.01 M, pH 5.6); highly porous hair with anhydrous ethanol; and hair of intermediate porosity with a 95:5 or a 90:10 solution of ethanol:water mixture. The differences in the wash kinetics obtained with these different solvents with a highly porous sample that had been contaminated by soaking in a cocaine hydrochloride solution (1 ug/ml for 1 hour) are shown in Figure 3. In previous reports (Baumgartner and Hill 1992; 1993) we showed that

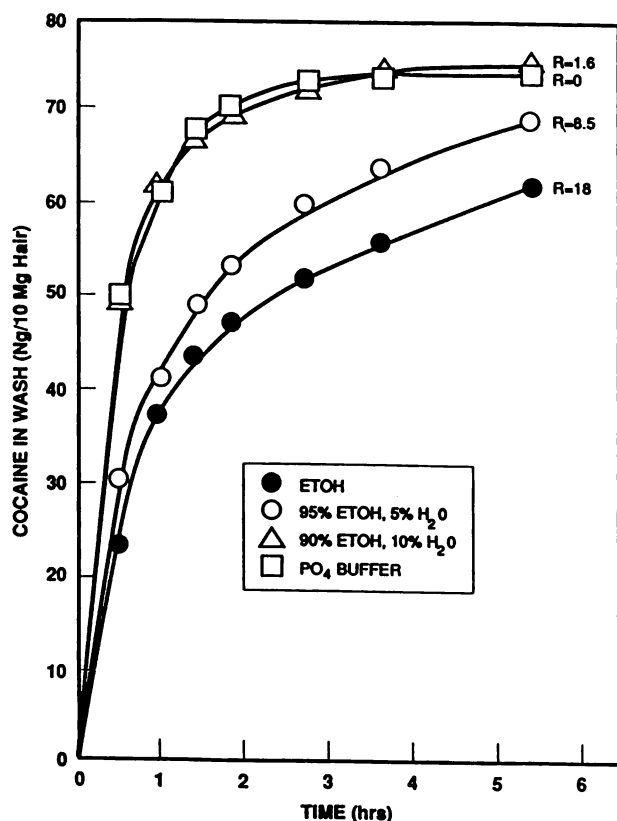


Figure 3. The effects of different wash solutions on the decontamination of porous hair at 37°C. The specimen had been contaminated by soaking in cocaine hydrochloride (1 ug/ml) for 1 hour. Listed R values represent the residual amounts of drug expressed as ng/10 mg. hair.

ethanol-based washes were far more effective than phosphate washes for discriminating between exogenous and endogenous drugs in porous hair. A typical example is shown in Figure 4. In this case we see that ethanol washes reach a plateau very quickly within 2 hours, and that subsequent phosphate washes release a large pulse of endogenous cocaine from the porous hair sample, while reaching a plateau more slowly after approximately 10 hours. Residual endogenous cocaine is removed only by digestion of the hair specimen (not shown in Figure 4).

In situations where estimates of drug dose are not critical (*e.g.*, preemployment testing) we prefer, for reasons of added safety, to use the harsher phosphate buffer washing for all hair specimens. Because of the hair-swelling properties of water, which are not present with other solvents, phosphate washes can remove substantial amounts of endogenously deposited drugs from porous hair.

Preliminary to the phosphate washes, hair was also given a 15-minute wash with ethanol to ensure that oils or greasy hair applications did not diminish the efficacy or influence the kinetics of the wash procedure. We prefer to degrease hair with ethanol rather than with detergent, since the latter can interfere with the RIA (radioimmunoassay) of the wash solution.

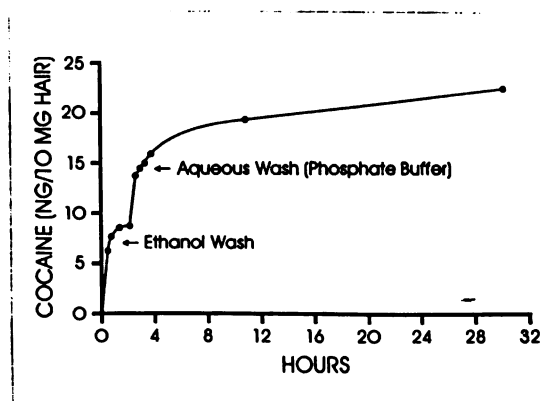


Figure 4. An example of the action of ethanol and water on cocaine-positive, treated (porous) hair.

With the extended wash procedure, the phosphate solutions were withdrawn at convenient time intervals and replaced with fresh solution. Contact times of at least 30 minutes per interval should be allowed to ensure maximum hair swelling. In the truncated procedure, hair was washed for 15 minutes in ethanol and then 3 times for 30 minutes in phosphate buffer. Washing was performed at 37°C with shaking of the test tubes at 100 oscillations per minute; 20 mg of hair in a 12 x 75 mm test tube were washed in 2 ml of phosphate buffer. Aliquots of the wash solution were analyzed for their drug content and the results used for constructing the wash kinetic curves.

Extraction of Drugs From Washed Hair

The most effective method for measuring the amount of drug remaining in washed hair was to dissolve the hair, for only by dissolving the specimen can one be certain that all the residual material entrapped in the protein matrix has been released. If solvent extraction procedures are used, complete extraction of analyte cannot be guaranteed, since extraction efficiency greatly depends on the physical properties of the hair (*i.e.*, whether it is thick and intact or thin and damaged). Of course, the procedure used for the dissolution of hair must be extremely mild in order not to cause decomposition of analyte.

For mass spectrometric analyses of 5 drugs (cocaine/ benzoylecgonine, PCP, morphine, methamphetamine, and marijuana), we chose a mild enzymatic digestion procedure at a pH of 6.2 (*i.e.*, at a pH at which cocaine does not hydrolyze to benzoylecgonine). No special precautions need be taken to measure the other metabolites in hair (*e.g.*, Δ -9-carboxy-THC, monoacetylmorphine, and amphetamine). The digestion of the hair specimen is as follows. To 10 ml of 0.5 M Tris buffer, pH 6.2, add 60 mg dithiothreitol, 200 mg sodium dodecyl sulfate, and 209 units Proteinase K. Add 2 ml of this solution per 20 mg of washed hair, the amount of hair typically used for hair analysis. Place in 37°C shaking water bath and shake at 80 - 100 oscillations/min overnight (16 - 18 hours); remove from water bath and mix. Centrifuge to remove the melanin pellet; collect the supernatant for extraction and preparation for GC/MS procedures.

The resulting protein solution, minus the melanin fraction, is analyzed by sensitive negative or positive

chemical ionization mass spectrometric techniques in current use for blood analysis (Foltz *et al.* 1980a; 1980b; 1983; Taylor *et al.* 1987). In the case of marijuana, the method of Foltz (Foltz *et al.* 1983) had to be adapted to ultrasensitive GC/MS/MS procedures which were developed to measure the low levels of Δ -9-carboxy-THC in hair (Hayes *et al.* 1991).

It should be recognized that chemical conditions which lead to the dissolution of one of the most resilient protein structures (hair) are highly deleterious to the most sensitive of proteins (antibodies). Consequently, the digest used for mass spectrometry is quite unsuitable for RIA analysis. In its place, Psychomedics uses its proprietary (patent pending) hair analysis procedure which requires 0.5 - 1.0 mg hair per analyzed drug. Preliminary RIA screens may also be done by several published RIA procedures (Baumgartner *et al.* 1979; 1981; 1982; Valente *et al.* 1981).

Qualitative Wash Kinetic Analysis

Our experience with over 10,000 positive hair samples has shown that wash kinetic data can be grouped into five broad categories. These are shown in Figure 5 and are described below.

1. No drugs are found in the wash solution but the amount of endogenous drug released by the hair digest is large. This situation arises with uncontaminated strong hair from drug users (not shown in Figure 5).
2. Kinetic Curve D: Only the first alcohol wash, but not the phosphate buffer washes, contains detectible amounts of drugs. The alcohol wash is believed to remove only adsorbed contamination from a shallow surface layer. Such rapidly attained plateaus in the wash kinetics occur in case of strong, externally contaminated hair (*i.e.*, no significant amounts of endogenous material are removed by the phosphate buffer washes). The pulse of drug released upon digestion of the hair is very large compared to the total amount removed by the ethanol wash. It is the combination of the attainment of the plateau and the magnitude of the pulse which allows one to discriminate with certainty between endogenous and exogenous drugs.

3. Kinetic Curve C: Both the alcohol and the phosphate washes contain drugs. The latter removes both deeper-lying exogenous drugs and some endogenous material. A near plateau is attained with the truncated wash procedure. Once again, the pulse of drug released from the digested hair is large compared to the total amount found in the phosphate washes. These wash kinetics are obtained with mildly porous hair.
4. Kinetic Curve B: The wash kinetics of the truncated method have not attained a plateau and the amount of drug found in the phosphate buffer washes may exceed the amount found in the hair digest. These cases are encountered with weak hair or with hair which, as a result of perming, dyeing, etc., have been rendered porous, causing thereby a considerable loss of endogenously derived drugs to the phosphate buffer. Such hair is best washed with milder wash solutions. However, irrespective of the solvent used, one is still able to identify endogenous drugs by the considerable pulse of released drug following hair digestion.

5. Kinetic Curve A: This represents the case of contaminated hair from a nondrug user. Here the relationship of the washout kinetics to the drug pulse released upon digestion is quite different from that of endogenous drugs: the kinetics are sharply curving (as demonstrated by the specific examples in Figures 1 and 2). The pulse of drug released upon digestion of hair is zero or insignificant relative to the amount of material removed by washing.

On the basis of these data a model for the sequestration of drugs by hair has begun to emerge. The model is based on the demonstrated resistance of hair to severely applied external contamination, on the ready removal of such contamination by various wash solutions, and most importantly on the inability of even harsh wash solutions to deplete hair of its endogenous drugs. The kinetics generated by the wash procedures are also highly significant for the model (*i.e.*, the rapid attainment of a plateau or near plateau followed by a large pulse of released drug upon the digestion of hair).

These data suggest the existence of two fundamentally different domains for drug sequestration in hair—an accessible and an inaccessible domain. Blood-derived endogenous drugs appear to be distributed over both domains, whereas exogenous drugs can only enter the accessible domain. The size of the accessible domain depends on the porosity of the hair and on the severity and nature (vapor *v.* solution) of the contamination conditions. The type of solvent used (swelling *v.* nonswelling) for applying the contamination or for washing is also important in the definition of the two domains.

It is also evident that the five drugs of abuse are not tightly bound to hair proteins for the drugs are readily removed by washing with a variety of solvents. Thus, the inaccessible domain appears to maintain its drug content not by stronger binding interactions but rather by structural sequestration. The rope-like macroprotein structures as well as the organization of hair into cellular domains offer many possibilities for such a model. Consequently, those drugs which enter hair easily are also easily removed.

This model, however, does not imply that the accessible domains are necessarily located only at the surface of the hair. It is quite possible that the accessible domains also penetrate deep into the hair structure in

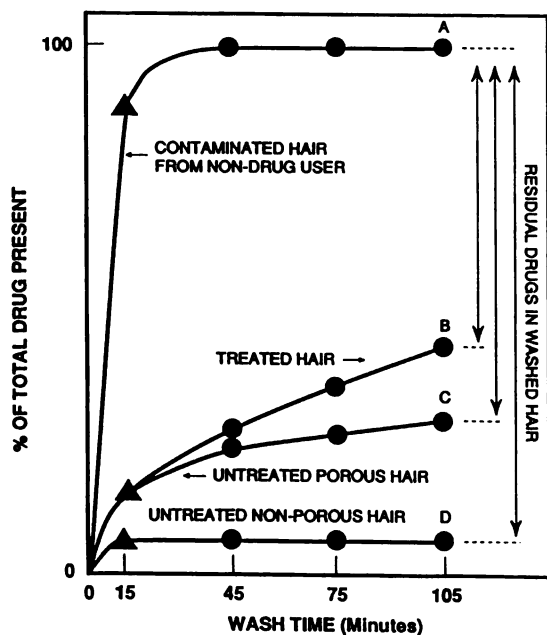


Figure 5. Wash kinetics of different types of hair from drug users and nondrug users. \blacktriangle , ethanol wash; \bullet , phosphate buffer wash.

pore-like fashion (*i.e.*, accessible domains may involve the spaces which are readily entered by water) causing the well-known swelling phenomena of hair. If these considerations are correct, then endogenous drugs may not be distinguished from exogenous drugs by cross-sectional drug-specific antibody staining of hair as has recently been proposed.

Quantitative Wash Kinetic Analysis

With the type of hair and type of contamination shown in Cases 1, 2, 3, and 5 of Figure 5, the wash kinetic results obtained with the truncated wash procedure are essentially identical to those obtained with the extended wash procedure (*i.e.*, both procedures approach a plateau rapidly). However, when a plateau is only slowly attained with the extended wash procedure, then the wash kinetic results of the extended and truncated (Case 4) procedures differ significantly. In this situation, which is the most frequently encountered wash result, we face the question whether the residual pulse of presumed endogenous drug remaining after three phosphate washes could have been depleted if the truncated wash procedure had been extended.

We developed three kinetic criteria for evaluating the probability of such an occurrence; one involves a retrospective and the others a prospective approach. In the prospective approach we are faced with the following question—given that the last wash (third phosphate buffer wash) contains X amount of drug, what is the probability that additional washes would have removed the residual drug in the hair? This obviously depends on the magnitude of the residual drug relative to that in the last wash and on the degree of curvature of the wash kinetics at the time of the last wash (*i.e.*, the extent to which a plateau had been approached). To evaluate this situation, we defined the extended wash ratio R_{EW} and the curvature ratio, R_C .

The extended wash and curvature ratios are defined as follows:

$$R_{EW} = \frac{\text{amount of drug per 10 mg hair in digest}}{\text{amount of drug per 10 mg hair in last PO}_4 \text{ wash}}$$

$$R_C = \frac{\text{amount of drug per 10 mg hair in 3 PO}_4 \text{ washes}}{3 \times \text{amount of drug per 10 mg hair in last PO}_4 \text{ wash}}$$

In the retrospective approach, we ask—having removed Y amount of drugs by all three phosphate buffer washes, what is the probability that the residual drug could have been removed by an extension of the truncated wash procedure? Here too, the answer depends on the relationship of Y to the residual amount in hair. The smaller Y is, relative to the residue, the smaller the probability. Again a high degree of curvature in the wash kinetics provides additional assurance that the residual drug would not be removed by an extension of the truncated wash procedure. The ratio evaluating this probability is termed the Safety Zone Ratio:

$$R_{SZ} = \frac{\text{amount of drug per 10 mg hair in digest}}{\text{amount of drug in all PO}_4 \text{ washes per 10 mg hair}}$$

The drug contained in the alcohol wash has been excluded from this calculation since this could assume any high value as a result of loose surface contamination. It is also important to realize that the presently defined kinetic criteria (particularly the numerical values) are only valid if the residual drug in the hair fiber is measured by a method that guarantees the complete release of the entrapped drug (*e.g.*, a digestion procedure).

The wash/kinetic ratios were initially evaluated with 700 hair specimens from known cocaine users. Our contamination experiments and field studies with a smaller number of opiate, methamphetamine, and PCP positive hair specimens indicated that the ratios apply equally well to the other drugs of abuse. Only marijuana is an exception in this regard. Here we found that the ratio method was rendered inaccurate by an unfavorable signal-to-noise ratio (*i.e.*, with marijuana the concentration of endogenously deposited drug in hair is too small in comparison to what can be deposited exogenously).

The percentage distribution of values for the 3 wash kinetic ratios is given in Tables 2, 3, and 4. The lowest value in each table (10, 0.33, and 1.3) is the cutoff value for the wash kinetic criterion. It should be noted that a value of 1.0 for R_C would indicate perfectly linear kinetics for the 3 phosphate washes.

Our field study showed that a small fraction of samples provides ratio values near or below the cutoff levels and that these samples are mainly those which had been cosmetically treated. When these samples were rewashed with a less-harsh solvent (*i.e.*, with the ethanol/water mixture) a shift to high ratio values occurs.

Table 2. PERCENTAGE DISTRIBUTION OF EXTENDED WASH RATIO VALUES*	
Extended Wash Ratio	Percentage of Total Samples
10 - 20	17.7
20 - 40	26.8
40 - 100	33.3
100 - 200	9.5
>200	12.6
*Extended Wash Ratio = $\frac{\text{Amount of Drug Per 10 mg Hair in Digest}}{\text{Amount of Drug Per 10 mg Hair in Last PO}_4 \text{ Wash}}$	

Table 3. PERCENTAGE DISTRIBUTION OF SAFETY ZONE RATIO VALUES*	
Safety Zone Ratio	Percentage of Total Samples
0.33 - 1.0	7.1
1.0 - 5.0	36.8
5.0 - 10.0	25.6
>10	30.5
*Safety Zone Ratio = $\frac{\text{Amount of Drug Per 10 mg Hair in Digest}}{\text{Amount of Drug Per 10 mg Hair in All PO}_4 \text{ Washes}}$	

Table 4. PERCENTAGE DISTRIBUTION OF CURVATURE RATIO VALUES*	
Curvature Ratio	Percentage of Total Samples
1.3 - 1.5	13.4
1.5 - 2.0	19.2
2.0 - 5.0	38.8
5.0 - 10.0	9.4
>10	19.2
*Curvature Ratio = $\frac{\text{Amount of Drug Per 10 mg Hair in 3 PO}_4 \text{ Washes}}{3 \times \text{Amount of Drug Per 10 mg Hair in Last PO}_4 \text{ Wash}}$	

Naturally, the certainty of a finding of drug use, as distinct from passive exposure, increases the further the wash kinetic ratios are removed from the cutoff values. With hair analysis one attains additional certainty by the requirement that a diagnosis of drug use is made only when all three wash kinetic ratios exceed their cutoff values.

The effectiveness and accuracy of the wash kinetic criteria were subsequently confirmed by our experience with over 150,000 hair samples. These yielded over 10,000 positive results from individuals who were either applicants for employment or participants in over 16 clinical field trials.

Identification of Drug Use by the Measurement of Metabolite

The identification of drug metabolites in hair specimens was used as an independent check on the validity of the wash kinetic approach for distinguishing between active drug use and surface contamination. We developed several procedures for the measurement of drug metabolites in hair. Wherever possible, we use the relative measure (*i.e.* metabolite as percent of total drug) rather than the absolute concentration of the metabolite as an indicator of drug use. The following metabolite procedures are applied on a routine basis in our laboratory.

COCAINE. Benzoylecgonine is the major metabolite of cocaine. For its measurement, it was necessary to develop a method of hair digestion which

proceeds at sufficiently low pH values to avoid the hydrolysis of cocaine to benzoylecgonine. As previously indicated, a pH range of 6.2 - 6.5 was found to be highly effective. To measure benzoylecgonine levels in small quantities of hair near the cutoff level of 5 ng cocaine/10 mg hair, it is necessary to use sensitive GC/MS/MS or ion-trap procedures (Harkey *et al.* 1991a). To achieve the required accuracy at these low levels, it is also necessary to include both deuterated benzoylecgonine and deuterated cocaine as internal standards. Failure to do this may have caused one laboratory to miss the presence of benzoylecgonine in hair specimens containing only low levels of cocaine (Harkey *et al.* 1991b). The low cocaine levels of this study also create considerable doubt that the six analyzed hair specimens came from heavy cocaine users as reported in that study on the basis of self-reports.

The ability to accurately distinguish between external contamination and cocaine use through benzoylecgonine determination has been confirmed by a number of studies (Baumgartner and Hill 1992; 1993; Cone *et al.* 1991; Kidwell and Blank 1992; Martz *et al.* 1991; Moeller and Fey 1991). The results from our own studies are listed in Table 5. The average values and ranges of the percentages of benzoylecgonine found by our procedure are comparable to those reported by the other investigators.

METHAMPHETAMINE. We have identified both methamphetamine and its metabolite—amphetamine—in the hair of methamphetamine users. The method is reported by Foltz (Foltz *et al.* 1980b)

Table 5. BENZOYLECGONINE AS PERCENT OF TOTAL COCAINE AND BENZOYLECGONINE IN HAIR WASHED BY TRUNCATED WASH PROCEDURE

Total Cocaine and Benzoylecgonine (ng/10 mg Hair)	N	Percent Benzoylecgonine	
		Average	Range
10 - 30	21	25	12 - 33
30 - 60	24	24	11 - 37
60 - 100	28	24	10 - 38
100 - 200	25	18	11 - 34
200 - 300	13	15	10 - 25
>300	17	14	9 - 30

and was modified for positive chemical ionization by GC/MS/MS.

The measurement of the metabolite is not only useful for distinguishing between external D-methamphetamine contamination and drug use, but is also effective in addressing the L-methamphetamine problem caused by nasal spray inhalants. External L-methamphetamine contamination is identified by the absence of its amphetamine metabolite. Passive exposure to L-methamphetamine, which poses considerable problems to urinalysis, does not play a role in hair analysis as such exposure results in hair levels below the cutoff level of 5 ng/10 mg hair. In addition, the L- and D-forms can be readily distinguished by comparison of the RIA and mass spectrometric data. Good agreement is observed between our RIA method and MS results with D-methamphetamine; but because of different antibody affinities, the quantitative GC/MS and RIA results differ five- to ten-fold when L-methamphetamine is analyzed by RIA with D-methamphetamine standards.

The percentage of amphetamine in hair relative to methamphetamine ranges from 3% to 20%.

OPIATES. A number of metabolic approaches can be used to distinguish between external contamination with opiates and opiate use. For one, morphine is a metabolite of heroin. In addition, we and others have reported the presence of another heroin metabolite, monoacetylmorphine (Goldberger *et al.* 1991).

However, the most convenient and sensitive metabolite procedure is based on the detection of morphine glucuronide in hair. This involves measuring the increase in the free morphine in hair as a result of glucuronidase treatment. The hydrolysis reaction is performed with 100 μ l gluculase (New England Nuclear) per 1 ml hair digest (10 mg hair/ml) for 3 hours at 37°C and pH 5.2. The percentage increase of free morphine resulting from gluculase treatment is shown in Table 6. Morphine glucuronide was found in the

Table 6. INCREASE OF FREE MORPHINE IN HAIR DIGESTS AFTER TREATMENT WITH GLUSULASE

Sample Number	Morphine Equivalents (ng/10 mg Hair)		Percent Increase (B-A/A)
	A. Before Glusulase	B. After Glusulase	
1	16	44	175
2	48	84	75*
3	15	36	140
4	10	16	60*
5	27	53	96
6	42	69	64*
7	9	22	152*
8	49	78	59*
9	36	64	76*
10	29	39	34
11	43	56	30*
12	7	15	114*
13	37	66	78
*Starred Samples Were Shown to Contain Both Codeine and Morphine.			

hair of all opiate users analyzed to date.

MARIJUANA. Because of the unfavorable signal-to-noise ratio of endogenous and exogenous cannabinoids in hair, we do not use wash procedures but only the presence of the metabolite Δ 9-carboxy-tetrahydrocannabinol to differentiate drug use from external contamination. We developed sensitive GC/MS/MS techniques for the detection of this metabolite (Hayes *et al* 1991). Δ 9-carboxy-THC was not found to be present in marijuana smoke.

PHENCYCLIDINE. No significant metabolites have been identified for phencyclidine in hair. Consequently, as with urine, only the starting material phencyclidine (PCP) is measured in hair. In this case we rely entirely on wash kinetic procedures to differentiate between contamination and drug use. Extensive contamination studies have demonstrated the effectiveness of this procedure.

Unrealistic Contamination/Decontamination Experiments

Judging from several recent reports (Cone *et al* 1991; Goldberger *et al* 1991; Kidwell and Blank 1992), a number of investigators appear to have misunderstood the theoretical basis of our wash kinetic criteria for distinguishing between drug use and external contamination. Nowhere do we claim, as these investigators report, that one 15-minute alcohol wash and three 30-minute phosphate washes can remove every type of external contamination, particularly the severe and highly unrealistic ones employed by these investigators (*e.g.*, soaking of hair specimens for 24 - 48 hours in concentrated drug solutions). This is clear even from our own data (Figure 2) in which hair of different porosities are bathed for 3 hours in a solution of cocaine hydrochloride—in our opinion a very extreme and highly unlikely case of external contamination. Here only the nonporous hair (Curve C) is completely decontaminated. Small traces of cocaine remain in the hair of intermediate porosity (Curve B), but the level here is below our cutoff of 5 ng/10 mg hair, whereas significant amounts of cocaine remain in the extremely porous hair after 90 minutes of washing.

What we do maintain; however, is that our three wash kinetic criteria clearly indicate that the hair specimens are contaminated (*i.e.*, that they are not from drug users). For example, the worst contamination case, shown in Figure 2 (Hair A), yields an extended wash

ratio of 1.2 and a safety zone ratio of .18 (*i.e.*, values which lie far below their respective cutoff values of 10 and 0.33).

In using our wash kinetic criteria, investigators must understand that the numerical values of the kinetic ratios apply only under the exact experimental conditions of our procedures. This pertains not only to the wash step, but also to the necessity of a 100% extraction efficiency for the measurement of the residual drug in the hair.

Finally, it is obvious that our wash kinetic criteria do not apply if the external contamination procedure is so severe as to result in the formation of control material (*i.e.*, material where the drug has been forced into the inaccessible domain of the hair specimen). Under such conditions only the metabolite criterion for exogenous drugs is applicable.

Our experience indicates that penetration of exogenous drugs into inaccessible domains does not occur in everyday situations. Whether this occurred with the hair samples that were soaked for 24 - 48 hours in concentrated drug solutions cannot be established from the published data. One reason for this is that the residual drug was not measured by a digestion method guaranteeing 100% extraction efficiency. However, regardless of what our wash kinetic approach would reveal about such hair specimens, such extreme soaking experiments cannot by any stretch of the imagination be viewed as realistic contamination scenarios. Furthermore, it can be readily shown on the basis of simple calculations and experiments that the exposure of an individual to conditions leading to significant external contamination of hair will also result in a positive urine test. However, in contrast to urine, where the result is indistinguishable from active drug use, hair analysis will return a result of "contamination" (*i.e.*, a result of "no drug use") on the basis of its metabolite and wash kinetic criteria.

CORRELATION BETWEEN HAIR LEVELS AND DRUG DOSE

Initially we investigated the relationship of drug dose and drug concentrations in hair with inbred mice (syngeneic, C57/BL6) in order to avoid the problem of variable responses due to biochemical individuality. We investigated by these experiments whether there was a linear correlation with dose or whether drug levels in

hair tended to plateau at higher dose levels.

We studied this relationship in animals with cocaine, morphine, PCP, and methaqualone and found not the slightest sign of a curvature in the dose-hair concentration relationship; linearity was maintained all the way to the lethal dose (LD_{50}) (Baumgartner *et al.* 1989). The linear dose correlation was subsequently confirmed with human subjects by the controlled administration of small quantities of cocaine (Harkey *et al.* 1991b) and codeine (Cone 1990). Here, a doubling of the administered dose resulted in a doubling of the concentration of the drug in hair. That this correlation is maintained for higher doses is suggested by the linear correlation between self reports of quantities used and the drug level in hair (Baumgartner *et al.* 1989). We have now demonstrated such a correlation for methamphetamine (Figure 6) and methadone (Figure 7). With methadone, hair levels are correlated with different dose levels which were administered without supervision. The relatively poor correlation with dose is largely caused by variable degrees of gastrointestinal absorption (see discussion of bioavailability below).

This linear correlation with dose is the basis for

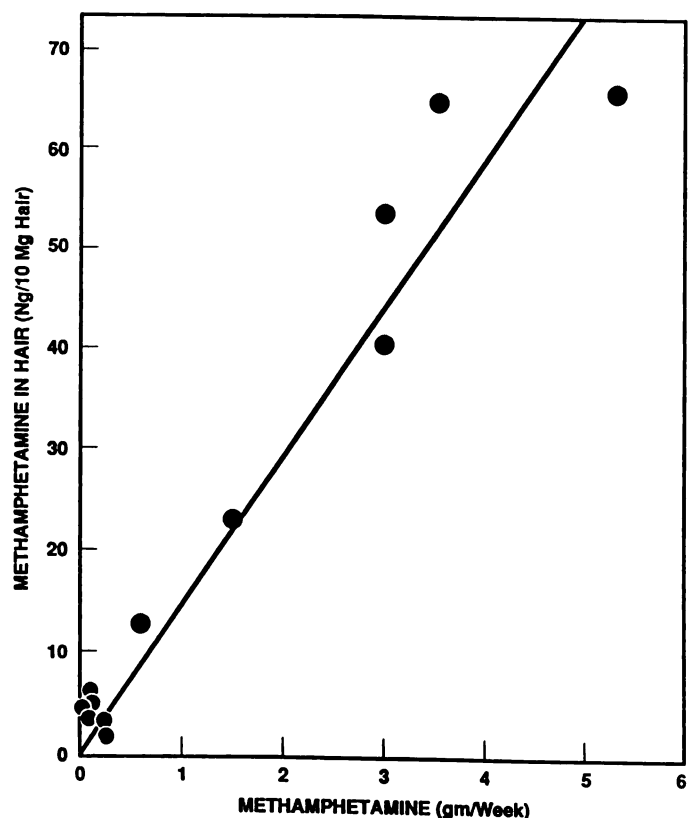


Figure 6. Correlation between self-reported amounts of methamphetamine use and methamphetamine levels in hair.

the ability to determine the pattern or relative change in drug use by segmental hair analysis. The linear correlation also suggests that hair continuously monitors various drug pools in the body, acting thereby as a probe or dosimeter of the bioavailability of the drug. This can be seen more clearly on recognizing that hair, like urine and feces, functions as an irreversible excretory pool. Thus, when the administered dose is doubled, the total amount of drug excreted into these pools will also double. Because of the sharply rising and falling excretion kinetics of drugs in urine, one random sampling of the urine excretion profile cannot be correlated to dose. Such a relationship will hold only upon integrating the total area under the urine excretion curve for the time required for clearance of the dose. This integration is automatically performed by hair as it monitors the endogenous drug pools.

When we now move from the individual to a population of drug users one encounters, of course, the important variable of biochemical individuality (Williams 1974). Accordingly, different people using the same amount of drug will deposit different amounts of drug into the hair. Harkey and Henderson (Harkey

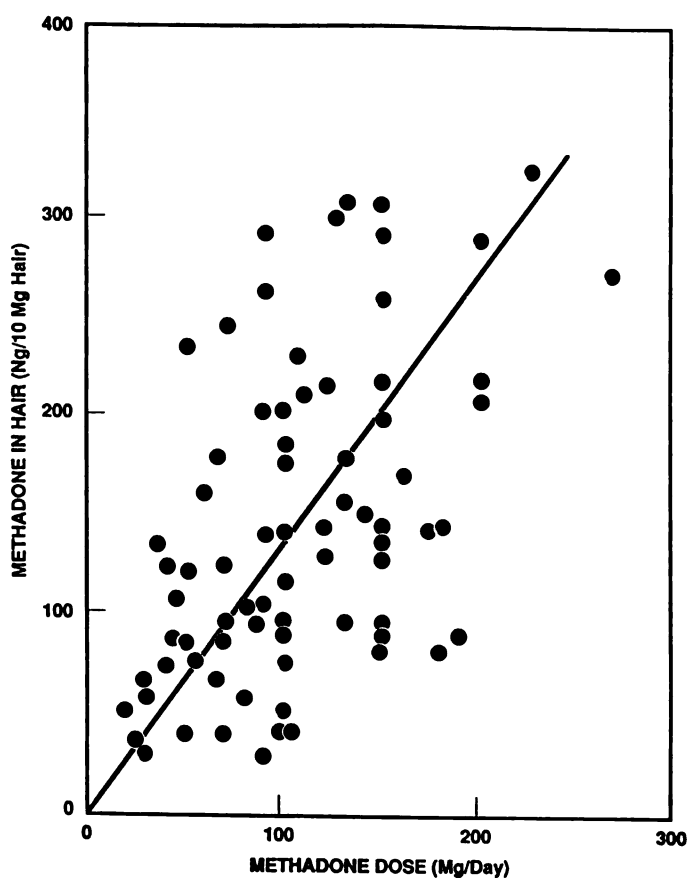


Figure 7. Correlation between methadone levels in hair and prescribed doses administered without supervision.

et al. 1991b) have attempted to obtain a measure of the biochemical variability for drug deposition by the controlled administration of low doses of deuterated cocaine. Their work, however, may be an overestimate of biochemical individuality since the subjects of their study appear to have had widely varying drug-use histories. This, in turn, is expected to result in different degrees of catabolic enzyme induction and, therefore in an increased spread in the rates at which the study participants metabolized the small fixed dose of deuterated drug.

Nevertheless, in spite of biochemical variability, we observed a correlation between the self-reported amounts of drug use and drug levels in hair. This is not the case for marijuana because of the previously mentioned methodological difficulties of distinguishing exogenous from endogenous material by washing. Obviously, such correlations improve with increasing dose range. Consequently, one can distinguish between heavy and light drug users. These two extremes naturally are separated by the gray zone of intermediate users.

Although it is clear that one cannot determine from hair analysis the exact drug dose, it is possible that drug dose is not always the most meaningful information in certain clinical situations. In fact, hair may provide a bioavailability index for a particular drug (*i.e.*, a therapeutic or toxicological index). That is, drug levels in hair may be more closely correlated with therapeutic effectiveness, toxicological symptoms, or addiction than with blood levels or dose. For one, the drug content of hair does not suffer from the peak and trough problems of blood measurements since the hair follicles continuously monitor the whole blood kinetic profile.

A therapeutic hair index more effective than blood levels has already been reported for haloperidol (Matsuno *et al.* 1990) and is currently being investigated by us for methadone. Because of the uncertainty of the degree of intestinal absorption of orally taken methadone, hair levels may provide a useful index of methadone bioavailability. Irrespective of the above, segmental hair analysis can give an accurate measure of patient compliance with a prescribed methadone medication schedule, particularly if this is initially administered under supervision, so that the patient through his original hair level becomes his own control during the unsupervised period.

Independent evidence for the ability of hair analysis

to measure the severity of drug use comes from studies comparing the efficiency of identifying drug users by unannounced urinalysis and hair analysis. Because of the fast excretion of drugs like cocaine it is generally recognized that the probability of identifying a cocaine user by unannounced urinalysis increases with increasing frequency of drug use. However, because of its wide window of detection, the probability of identifying drug users by hair analysis is largely insensitive to the frequency of drug use. Thus if the grouping of drug users on the basis of hair analysis into light, intermediate, and heavy user categories is correct, then a comparative study with unannounced urinalysis should show a greater number of urine positives in the heavy user group than in the light user group.

This situation was indeed observed in several blind studies with approximately 1,000 subjects from the criminal justice system who, at the time of their arrests, were tested for cocaine by hair and urine analysis (Mieczkowski *et al.* 1991). The urine tests were performed with a sensitive immunoassay procedure with a cutoff level of 300 ng/ml and hair analysis by the Psychomedics procedure. A subset of these results is shown in Table 7. These studies confirm the findings of an earlier investigation (Baer *et al.* 1991) where this trend was first demonstrated with a less sensitive urine test.

The drug use indices of hair analysis have also been used to characterize drug use in several different populations (*e.g.*, applicants for employment, arrestees, and patients in drug treatment programs). As expected, we see a far greater number of heavy drug users in the last two populations than in the preemployment group (Table 8).

NO EVIDENCE OF RACIAL BIAS

The question has been raised whether the hair test is biased with respect to race [*i.e.*, whether the structure (thickness, curliness) and color of hair, or possible differences in the rate of the biochemical processes involved in drug deposition could produce a statistically significant difference in drug levels in hair between racial groups]. Although the question is equally relevant for urinalysis, it appears not to have been investigated to date.

On theoretical grounds it appears unlikely that hair analysis, or for that matter urinalysis, should be

Table 7. COMPARISON OF EFFICIENCY OF DETECTION OF DRUG USE BY HAIR ANALYSIS AND UNANNOUNCED URINALYSIS IN LIGHT, INTERMEDIATE, AND HEAVY COCAINE USE CATEGORIES

Hair Drug Use Index	Cocaine in Hair (ng/10 mg)	Number of Positives		Urine Positives Relative to Hair Positives
		By Hair	By Urine	
Light	2 - 30	68	10	14.7%
Intermediate	31 - 100	27	11	40.7%
Heavy	>100	46	31	67.4%

Table 8. PERCENTAGE DISTRIBUTION OF LIGHT, INTERMEDIATE, AND HEAVY COCAINE USERS AS MEASURED BY THE HAIR DRUG USE INDEX¹ IN THREE DIFFERENT POPULATIONS

Population	Number Tested	Percent Positive	Percentage Distribution of Severity of Cocaine Use		
			Light	Inter-mediate	Heavy
Drug Rehabilitation	130	75	28.5	20.0	51.5
Arrestees	279	57	47.8	18.9	33.3
Workplace	7225	6.2	66.8	17.2	16.0
¹ Hair Cocaine Use Index: Light Use - 2 - 30 ng cocaine/10 mg hair Intermediate Use - 30 - 100 Heavy - >100					

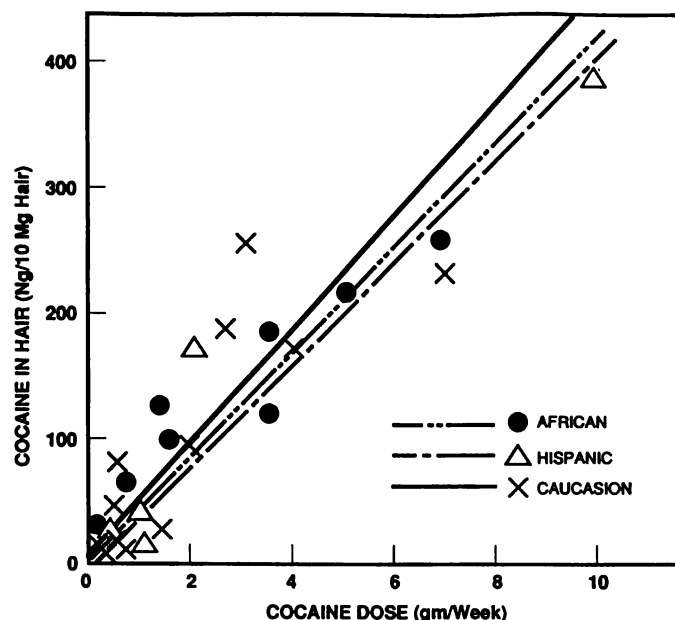


Figure 8. Correlation between self-reported amounts of cocaine use and cocaine levels in hair of Hispanic, African, and Caucasian ethnic/racial origin. This study was performed under conditions which favored accurate self-reporting of drug use.

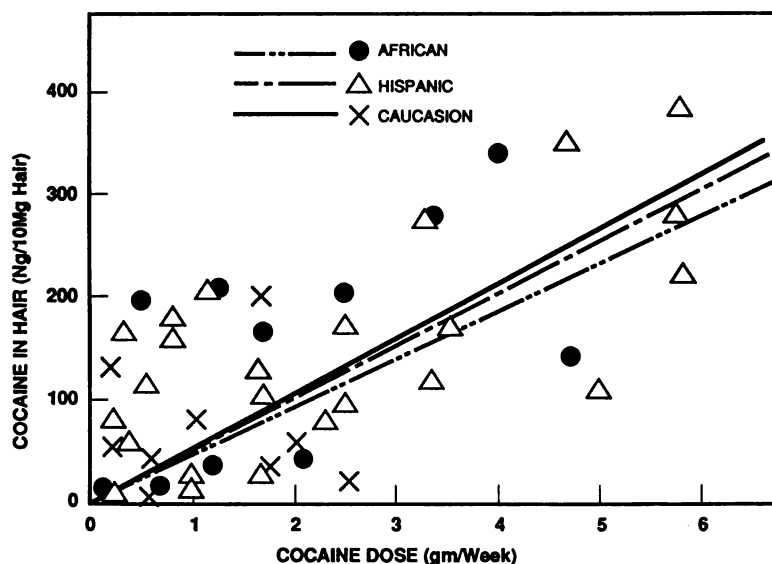


Figure 9. Correlation between self-reported amounts of cocaine use and cocaine levels in hair of Hispanic, African, and Caucasian ethnic/racial origin. The self-reports in this study were judged to be less reliable than those in Figure 8.

a digestion procedure, then the possible influence of hair thickness and color are excluded from the test. Thus, although solvent-based extraction procedures may remove drugs from strong hair (Asiatic) or fine blonde hair with different efficiencies, such differences are not possible when the hair is dissolved. The issue of hair color is also bypassed by a digestion method, since the drugs present in the colored melanin granules are not included in the analysis. The melanin granules are not extracted during digestion and are removed from the digest prior to analysis. Once again, such guarantees

cannot be given for solvent-based procedures.

It is also quite unlikely that the absorption, metabolism, and excretion of drugs into hair (or into urine) should differ between racial groups (*i.e.*, one would expect the wide spread in responses due to biochemical individuality within a racial group to be the dominant factor). This expectation is predicated on the well-established principle that macrostructural differences among races (*i.e.*, color, anatomical features) are unrelated to underlying common biochemical

are unrelated to underlying common biochemical mechanisms. This translates, in the case of hair, into the position that the inaccessible domains in hair are created by the biochemical properties of keratin shared by all racial groups. Similarly, as with urine, we believe that the excretory processes causing the transfer of drugs from blood into hair, are not dependent on macrostructural racial differences.

However, in light of public interest in the racial question, we decided to put the above considerations to the test. We adopted the experimental approach of Dr. Kidwell who first raised the issue of the possible effects of hair color and, by implication, race on the results of hair analysis (Kidwell and Blank 1992). In his small, inconclusive preliminary study with ten cocaine users, Dr. Kidwell investigated possible differences in the correlation between self-reported amounts of ingested drugs and drug levels in different colored hair.

We extended this investigation to 73 cocaine users (20 African, 29 Hispanic, and 24 Caucasian) with a focus on race rather than hair color since the latter could not have affected our results because of the discarding of the melanin fraction from our analytical process. The results were derived from three independent blind studies which used two different measures for estimating drug use from self reports. Only results from the two studies using the same self-report measure were pooled in order to minimize the statistical scatter that possible method biases could cause.

The results of these studies are shown in Figures 8 and 9. Regression lines were calculated for each racial group in each study. These were drawn through the origin in order to reflect our experience with numerous hair samples from guaranteed nondrug- using individuals which yielded analytical results indistinguishable from zero.

The results clearly show no statistically significant differences among the racial groups. Had this not been the case (*i.e.*, had any of the slopes of the regression lines been significantly different) then it would have been a relatively easy matter to apply a correction factor to the cutoff value for different racial groups in the case of hair because of its linear correlation between dose and drug levels. Such a correction factor is not possible for urine because of the absence of a dose correlation.

SEGMENTAL ANALYSIS

Head hair grows at a rate of approximately 1.3 cm/month. It continues to grow for about 1,000 days and then briefly enters a dormant period of about 2 - 3 months before falling out. Approximately 10% - 15% of head hair is at any one time in the dormant phase. In contrast to this, body hair grows at approximately the same rate as head hair, but it enters the dormant period very quickly. It then remains in its nongrowing phase for a much longer time than head hair before falling out.

These differences in growth characteristics make head hair, but not body hair, suitable for determining the pattern of an individual's drug use over a given period of time (*i.e.*, whether it is increasing, constant, or decreasing). With head hair one simply cuts a lock as close as possible to the scalp and then cuts it into segments corresponding to the time frames of interest. For example, to establish what the drug use was during the last month, one analyzes the first 1.3-cm segment from the root. The drug use which occurred during the second month is determined from the analysis of the second 1.3-cm segment located 1.3 - 2.6 cm from the scalp and so on. Hair grows at approximately 1.3 cm per month (Saitoh *et al.* 1967). This approach cannot be used for body hair since a harvest of 1.3 cm of body hair contains only a very small fraction of hair that has grown during the last month. Most of the hair in the body hair sample will be older, its age spanning a period of months to years.

As stated previously, our ability to accurately determine the relative changes in drug use during a given time is based on the linear relationship between dose and drug level in hair for a particular individual. However, a number of additional practical and theoretical factors must be considered before the results of segmental analysis can be accurately interpreted.

On the practical side, it is important to carefully clean the hair of external contamination so that only the material in the inaccessible domain in the hair shaft is measured. Insufficient attention to this practical detail may have given rise to the notion of a "wick effect" (*i.e.*, the postulated smearing-out along the hair shaft of endogenous drugs by daily cosmetic practices).

There is no experimental evidence for this suggestion; to the contrary, there is an abundance of data which contradicts the postulated longitudinal mobility of drugs entrapped in the inaccessible domains of hair. For example, this has been shown by us with numerous subjects who have ceased drug use, by demonstrating that the abrupt drop in the drug content of their hair is not smeared out by daily hygiene (Baumgartner and Hill 1992; Siegel 1992).

These observations have been confirmed by several independent studies in which individuals were administered a small bolus of drugs under controlled conditions. These drug pulses were tracked for many months in the hair of these individuals (Harkey *et al.* 1991a; Sato *et al.* 1989). In the cocaine study of Harkey and Henderson, some of these pulses disappeared over time. However, simple inspection of a thin hair strand will suggest that this was more likely to have been caused by loss across the hair strand than by longitudinal smearing out. Because of the small dose of administered drug (approximately one line of cocaine), the analytical signal of the drug pulse in hair must in many cases have been close to the detection limit of the analytical procedure. Consequently, the signal could easily have dropped below the detection limit as a result of cosmetic treatment of the hair in the intervening period.

Finally, the effects of dormant head hair must be considered in the interpretation of segmental hair analysis data. A typical example of this effect is shown

in Figure 10. This is the segmental analysis of an individual with a relatively constant cocaine habit 2 - 5 months prior to the collection of the hair specimen, yielding a drug level of approximately 200 ng/10 mg hair. Two months prior to sampling, this individual was referred to a drug-free environment. If drug use had completely stopped at the 2-month mark, one would expect the next 1-month segment to still present an "echo" of the previous drug use since 10% - 15% of the then growing hair would have entered the dormant phase. This would correspond to a drug level of 20 - 30 ng/10 mg hair. Consequently, the actual 26 ng/10 mg level found in this segment must be attributed to dormant hair and not to a small amount of drug use during the 1- to 2-month period. Experimental evidence shows that dormant hair is then lost at an approximately exponential rate, making its effect insignificant in the most recent (0 - 1 month) segment.

We have demonstrated the generality of these quantitative aspects of the dormant hair effects with several different collaborators (Baumgartner and Hill 1992; Siegel 1992). One of these studies was performed with patients from the Schick-Shadel organization, where cessation of drug use subsequent to entry into the rehabilitation program was documented by several measures. Hair specimens were obtained from these patients at the time of entry into the program and after 6 - 8 weeks of aversion therapy. A hair specimen corresponding to 4 weeks prior to entry was analyzed and compared to a segment corresponding to the last 2

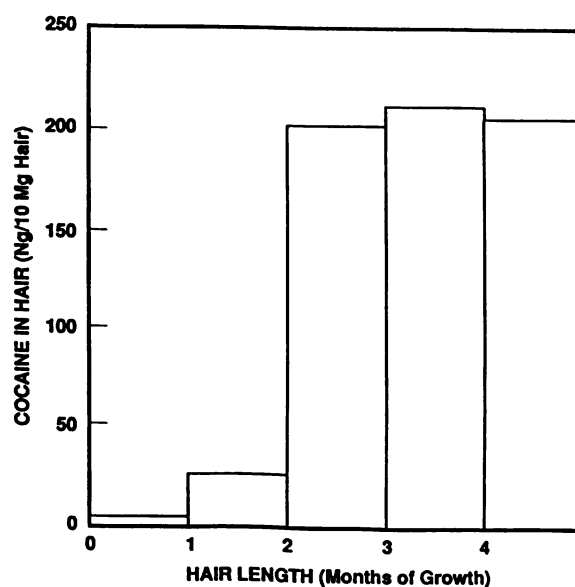


Figure 10. Segmental hair analysis, illustrating the effects of dormant hair. The individual in this study ceased drug use at the 2-month mark. Drug residues in the 0- to 1-month and the 1- to 2-month hair segments are caused by dormant hair.

Table 9. DORMANT HAIR EFFECTS IN HAIR ANALYSIS FOR DETERMINING COCAINE USE

1st Sample ¹ (ng Cocaine/ 10 mg Hair)	2nd Sample ² (ng Cocaine/ 10 mg Hair)	Time Between 1st and 2nd Samples (Months)	Dormant Hair Effect (%) (2nd/1st x 100)
32.5	0	1	0
264	2.5	2	0.95
56	0	2	0
56	0	2	0
57	0	2	0
88	0	2.5	0
317	3.3	2	1.0
101	0	2	0
113	0	1.5	0
115	0	2	0
193	2	2	1.0
41	0	2	0
286	8.3	1	2.9
168	3.8	2	2.3
¹ 1st Sample Was a 1.3-cm Section.			
² 2nd Sample Was a 0.6-cm Section.			

and compared to a segment corresponding to the last 2 weeks of therapy. The results in Table 9 show clearly that dormant hair effects do not interfere with our ability to demonstrate cessation of drug use. An essentially 100% drop in the drug content of hair is evident during the last 2 weeks of therapy. Also, since patients frequently washed their hair during the treatment period, it is evident that the abrupt drop in the drug content in these hair samples is inconsistent with the postulated wick effect.

CONCLUSION

Hair analysis has several advantages over urinalysis with respect to convenience, safety, and effectiveness. It can be collected under close supervision without

embarrassment. The solid hair specimen is not susceptible to subversive activities (*i.e.*, it cannot be tampered with by such means as spiking). Hence difficult-to-document security procedures at the collection site, during transportation, or in the laboratory are not as critical to hair analysis as to urinalysis. Hair can be matched to an individual by visual or microscopic examination or DNA analysis. And chain-of-custody problems can be readily overcome by the collection of a second specimen. The specimen can be shipped and stored indefinitely without refrigeration.

We have developed several independent measures to ensure the highest level of safety for hair testing. In particular, hair analysis is far less susceptible than urinalysis to evidentiary false positives caused by passive

drug exposure. In part this is due to the temporal overview provided by hair analysis and partly by a choice of cutoff levels which are set at a much higher level than urine relative to their maximum reported levels.

With respect to evidentiary false positives due to external contamination, hair also enjoys a number of advantages. In contrast to the lungs and intestinal tract, hair offers a highly resistant barrier to entry by external drug contaminants. And, if penetrated, the results of hair analysis are not confounded as with urinalysis by the generation of metabolites. Furthermore, the likelihood of such occurrence is greatly reduced by conventional hygienic practices, and by the application of highly effective wash procedures along with the three wash kinetic criteria. Additional safety is provided by the ability to collect a second hair specimen which can be subjected to even more extensive wash procedures, evaluation by methylene blue staining, and metabolite analysis by ultrasensitive GC/MS/MS procedures. The agreement of the analytical result of the second specimen with that of the first specimen affords another criterion for identifying external contamination, since such contamination tends to be highly variable.

Hair analysis can distinguish between external contamination and drug use; urinalysis cannot do this. The results of hair analysis are therefore reported as indicating: (1) no drug use; (2) drug use; or (3) external contamination by drugs. This trilevel reporting has considerable advantages since some organizations cannot tolerate even contact with drugs or drug users (e.g., the criminal justice system, high security occupations, laboratory personnel of drug testing laboratories). In one widely publicized case (Washington, DC, TV program), this distinction in the reporting of results was misunderstood and/or misrepresented.

The wide window of detection (from months to years) makes hair analysis a complementary test to urinalysis' short term information on drug use. In addition, hair analysis provides a measure of the severity of drug use (heavy, intermediate, or light) as well as information on the pattern of use (increasing, decreasing, or constant). Because hair can be collected under close supervision, it is not subject to the many evasive maneuvers of urinalysis such as temporary abstention prior to a scheduled test and sample manipulation.

As a result of these advantages, hair analysis is far more effective at identifying drug users than urinalysis.

This is true not only for scheduled (i.e., preemployment) testing but also for unannounced testing. Most importantly, hair analysis increases the level of comfort and confidence of both the implementors and subjects of drug testing, and for this reason its use as a backup test is also of great value for urinalysis.

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Dealing with Decomposed Remains: Selection of Toxicological Specimens

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The death investigator (whether he be a medical examiner or a coroner) presumably is diligent and strives to the best of his abilities and available resources, to certify a particular death correctly and scientifically. The autopsy examination is only one aspect of the investigation. It is, many times, of less importance than the circumstances or clinical history gleaned from medical investigators, police, family, medical records, or other witnesses. Without this information the autopsy findings cannot be put in proper perspective. The generation of other data is also essential in the quest for an accurate determination of the cause and manner of death.

Especially in light of the ubiquitous abundance of legally prescribed medications and illegally manufactured drugs of abuse, no single datum is as important as the toxicologic report. The development of a professional relationship with the toxicologic consultant is essential, whether he be physically within the same building or at a laboratory located in another state. Accurate and interpretable results can only be generated if there has been prior and ongoing communication between the prosecuting forensic pathologist and the director of the toxicology laboratory. This communication must be two-way. First the toxicologist should give clear instructions as to the type and quantity of each individual specimen that is to be obtained, as well as how the specimens are to be labeled, stored, transported, and documented. These guidelines will provide that the best possible specimens, with complete chain of custody, are received in the laboratory. In addition, the forensic pathologist must give the toxicology laboratory as much information as is available to him, after the examination has been completed.

Clearly, a general or routine screen for every possible drug and poison cannot be attempted. The pathologist must assist the chemists in focusing their analyses. Transmitting information generated by the death investigators, such as any known medications that the individual had been taking, or the specific drug of abuse that is suspected is crucial. The state of the body should also be indicated. This will give a clue as to the quality of the specimens. The forensic pathologist can request that only certain drugs be looked for in order to answer a specific question surrounding the autopsy examination. This would ensure that the valuable resources of the toxicology department or small samples are not wasted. It is also useful to the toxicology consultant to have some idea as to whether the results to be generated will be instrumental in certifying the cause of death, or if a cause of death has already been determined from the autopsy examination.

As with most things in life, and particularly in medicine and science, the quality of the end product is rarely dependent on a single individual. There is no doubt that communication greases the machinery of decision making and diagnosis.

THE IMPORTANCE OF TOXICOLOGY

The results of the toxicology laboratory are utilized at various levels of the death investigation. At the top of the list would be the ultimate determination of the cause of an individual's death. Depending on the jurisdiction involved, the primary determination of a death as being drug related can be up to 30% of all cases. Without the timely, accurate, and diverse tests that are currently available through tried and true

chemical techniques, and the modern computer-assisted analysis, a vary large proportion of deaths which come to a medical examiner's or coroner's attention would not be certified accurately.

A second important use for the results generated from the toxicology laboratory, would be in determining if substances found in the body had any contribution to an individual's death. Two common examples would be the detection of ethanol in a motor vehicle driver or a pedestrian sustaining injuries leading to their death. These results might help answer the question "How did the accident occur?" This is different than answering the question for the death certificate, "What caused the death?" For a pedestrian, the possibility of an incapacitating level of alcohol that may have put that person in the position of danger he would otherwise not have been in. Other important contributory conditions to the primary cause of death would be in a suicide where detection of such drugs as ethanol, cocaine, PCP, or LSD may give comfort, and a better understanding of the act to the loved ones that are left to deal with the death. Along those lines, drugs and alcohol that may be found might help a prosecuting or defense attorney elucidate the behavior of the deceased just prior to his death. A review of a toxicology report, indicating one or several different drugs, and without any other knowledge of the individual, may give clues as to a medical condition or lifestyle that person may have been involved with. Finding phenobarbital and carbamazepine may be the only clue that the individual suffered from a seizure disorder. The discovery of antidepressant medication may, for example, give a clue as to the manner of death and refocus or even generate a deeper investigation into the circumstances surrounding the individual's demise. Detectives armed with list of specific drugs, may find formerly silent witnesses becoming loquacious.

Police and scene investigators can also be extremely valuable in the death investigation by recovering any empty or filled prescription vials. The label, even if only partially intact, contains a wealth of information valuable to the police themselves and ultimately the forensic pathologist and the toxicologic consultant. The contents, if known, will help focus the analysis and, if unknown, may be available for comparison with substances found in blood and body fluids or other known resources. Prescription bottles were instrumental in discovering Tylenol laced with cyanide, one of several incidents which have become media fodder in the last

several years. Tampering may be individual for homicidal purposes or directed at a corporate entity for economic or revenge motives.

POSTMORTEM CHANGES

The commencement of irreversible cardiorespiratory arrest, or more simply death, starts a regular and predictable change in the human body as well as any other living mass of protein, carbohydrate, and fat. Although regular and predictable, the onset and detectability of each stage can be quite variable depending on the environment. The most important aspect of the environment is temperature. A second important factor is the presence or absence of other living organisms that may accelerate any or all of the stages of postmortem decomposition.

Prior to the ultimate fate as described in Genesis 3:14-19 ".... for dust you are and to dust you will return", numerous physical and chemical events affect the body. These processes can be viewed as the penultimate recycling project. Nature reclaims and renews those biochemical resources of matter and energy that are temporarily on loan to us.

In the early postmortem period or fresh stage, the two most widely talked about changes begin immediately after death. The physical change is livor mortis, and the chemical alteration is characterized as rigidity or rigor mortis. These have been the pillars on which postmortem intervals or time since death determinations have been precariously built. There still is no reliable individual or group of subjective or objective findings that can determine the postmortem interval. Before educated guesses can even be attempted, brackets must be drawn. These consist of when the individual was reliably last known to be alive and when he was found dead. Within these brackets such gross observations of livor and rigor mortis can be utilized, as well as the semiscientific recordings of algor mortis or postmortem cooling, and vitreous potassium measurement to cone down toward the exact time of death. Forensic entomology has come a long way in helping to narrow the postmortem interval in the later stages of decomposition. There are other esoteric attempts at identifying a reliable marker for postmortem interval determinations such as evaluations of cecal transmigration of bacteria, pH measurements of cardiac blood, and measurement of 3-methoxytyramine in the brain.

Livor mortis is the physical settling of blood in the vessels due to gravity. The blood will settle in the dependent portions of the body and may actually be visible in severely ill individuals with inadequate cardiac output causing venous stasis or peripheral vasodilatation sometimes seen in the shock state. In Caucasian individuals this is generally visible within 2 to 4 hours through the skin as a blue-gray (livid) color. It may be difficult to see in the Negroid race even after several hours. Areas of the body in tight contact with some surface or due to pressure on the skin by clothing or another object, may remain blanched because the capillaries are unable to fill with the settling blood. If the blood remains in this liquid state and the body is moved, the blood will resettle toward gravity potentially in the opposite side of the body. In the transition between the liquid and gel state, there may actually be livor on both sides of the body if it has been moved. Between 6 and 12 hours the blood will be less fluid and the lividity will not shift with changing body position. It is then said to be "fixed." Prominent livor in thin or highly vascular areas of the body may form small red blisters (Tardieu spots).

Rigor mortis is a chemical change within the myocytes. Due to the lack of oxygen lactic acid is produced which is theorized to either cause the cytosol to be transformed from a liquid to a gel state, and/or the enhanced cross linking of the actin and myosin fibers. This causes stiffening, to be contrasted with contraction, of the muscles. This is more discernable in the smaller muscles in the early phase, particularly those of the hands and face. The larger muscle groups become stiffer around the elbow and knee joints over time. It is generally held that rigor is detectable and ultimately reaches its peak within the first 12 hours after death, lasting approximately for 12 more hours and then ultimately waning over the third 12 hours. Since this is a chemical process, it will be accelerated by heat and retarded by cold. The body temperature at the time of death will also affect the development of rigidity. If the individual had been febrile, having a seizure, or in some heavy physical activity just prior to death the rigidity would develop rapidly. Also the quantity of muscle is also an important factor.

In temperate climates and moderate environments another early discernable change in the body is greenish discoloration of the right and then the left lower quadrants of the abdomen. This is due to a large concentration of bacteria that begin to multiply and

utilize the surrounding tissues as their culture medium. Additionally, even if the eyelids are closed the cornea will become progressively more opaque over this first 24-hour period. Further, the intercellular bridges will begin to break down and with ever decreasing amounts of tangential pressure, the layers of skin will slip upon one another.

With the onset of the changes of skin slippage the early decompositional stage begins. Autolysis, the breakdown of cells and organs because of their intrinsic enzymes, is a minor component. The internal organs become more liquefied as the process of putrefaction accelerates. This is the major element of decomposition caused by endogenous and environmental bacteria. As the tissues are breaking down the blood is also decomposing and leaches out of the blood vessels. This can be seen through the skin as green to violet, serpiginous and arborizing vibices, particularly of the upper extremities. Internally, the intimal surfaces become deeply stained with hemoglobin. The organs will progressively soften.

With progression into the moderate to advanced stages of decomposition, the individualizing features begin to distort. This progression is extremely temperature dependent, ranging from hours in the hot humid climates, to days or even weeks in cooler, drier environments. There is green to black discoloration of the skin particularly of the face and digits. There may be alternatively very moist areas, particularly centrally, with the distal areas dry and even mummified. As the putrefactive bacteria continue to exponentially multiply, they produce methane and sulphide gasses causing bloating of the soft tissues of the face, the lips, the neck, chest, abdomen, penis, and scrotum. At this stage visual identification and even determination of race may be very difficult. Internally the organs continue to liquefy. As the pressure increases, caused by the ever increasing bacterial gases, fluid can be forced from the oral, nasal, and rectal orifices. These dark red-black purge fluids, innocent signs of the progression of decay, may cause great consternation in inexperienced investigative personnel. An individual who had died a simple, natural death 5 or 6 days prior, may appear to have been involved in some heinous crime. Conversely, because of the overtly unpleasant odors and potentially frightening appearance of a moderately to advanced decomposed individual, true subtle and sometimes even gross injury may easily be overlooked. By their very nature and because the examination of the decomposed human is

so distasteful, these are the cases that require even more intense postmortem observation. Although subtleties may be consumed, quite obvious injuries and natural disease processes can still be identified.

The internal organs will ultimately continue to liquefy until they become amorphous. If the remains are still isolated from insect populations, the skin will probably remain intact and ultimately mummify. The putrefactive and decompositional phases are accelerated with the deposition of fly eggs which undergo instar larval growth phases. They will readily and rapidly consume nonkeratinized tissue. Other waves of insects, such as beetles, with different enzyme systems in their saliva will consume skin, tendon, and ligaments. These scavengers will ultimately lead to complete skeletonization. Larger animals such as carnivores may also accelerate loss of tissue and lead to dismemberment and disbursement of the remains over a wide geographic area.

There are other environments that will cause somewhat different postmortem changes. In an environment that is very dry, dehydration of the skin and even internal organs (if there is connection with the outside environment) may be the predominant alteration, preventing the autolytic processes and retarding the bacterial putrefaction. The skin turns dark brown and leathery in appearance. Facial features, although contracted, may be fairly well-preserved. Also depending on the changes of the environment, focal or complete loss of internal organs consistent with putrefaction and/or larval infestation may be contrasted with remarkably well-preserved, mummified (dehydrated) external features.

Another alternative environment would be a body immersed in fresh or salt water for an extended period of time. There is rapid bloating and putrefactive change there may also be significant external loss of tissue and marine animal artifact. If the moisture, temperature, and pH environment are in the right proportions, hydrolysis of the subcutaneous fat may develop, forming adipocere. This waxlike encasement may actually preserve the internal organs far beyond what one would expect.

Death of a fetus within the uterus is yet another environment. These dead fetuses can be retained a significant period of time such that, when delivered (if the environment has remained sterile) there is extensive

discoloration and tissue maceration caused by autolysis. Essentially the fetus is dissolving. In the earlier stages this is not to be confused with a scalded infant.

In other environments there may be significant changes of the body including embalming and/or burial. All routine samples may be available including blood, but a sample of the embalming fluid should be given to the toxicologist so he can match the "artifact" chemicals that would be detected in blood and tissue sample analysis.

Severely charred individuals are also somewhat unique. The blood may be completely congealed to almost a dust consistency. There may be extensive heat coagulative necrosis and protein denaturing of the other organs. If there is enough charring and dehydration the brain, liver, and kidney may not be available.

SPECIMENS

The collecting of blood, body fluid, and human tissue for toxicologic analysis is relatively straightforward. Even in the realm of decomposition, by applying the tenet that "one takes what one can get," reasonable and interpretable data can be obtained. With ever-improving techniques, more diverse and smaller quantities of tissue can be analyzed with great success. This includes alternative "tissues" such as larval and adult forms of insects associated with the remains.

In the fresh and intact body where little decomposition has occurred, standard specimens can easily be secured. The most important is obviously blood. There is the ongoing argument of collecting peripheral as opposed to central blood. Again, whatever is available should be collected. If needed, peripheral and central samples can be collected separately. The importance of this is that cardiac blood can possibly be "contaminated" by the leaching of drugs or medications from the myocardium itself, lung tissue, liver, and gastric contents. Sequestered blood such as a subdural hematoma is a snapshot of the toxicologic status of the person at the time he was injured and is a vital specimen. This is important in a patient with significant survival. Ethanol is especially looked for because it requires circulation through the liver to be metabolized.

A second valuable fluid which should be collected whenever possible is vitreous humor. Electrolytes, glucose, and drugs can readily be detected and measured.

Urine, gastric contents, and bile should also be routinely collected for at least qualitative identification of the presence and use of certain drugs or medications. Depending on the toxicology laboratory organs such as brain, liver, and kidney can also be routinely submitted.

If blood is available it should be put in a glass container and mixed with sodium fluoride and oxalate powders. This will inhibit the production of ethanol. It is important to know that bacteria proliferating in the early postmortem stage and throughout the putrefactive process do indeed produce ethanol. It is generally accepted that postmortem decompositional blood ethanol levels can be seen up to approximately .06%, however, levels of .10% to .20% have been occasionally reported.

As putrefaction progresses and blood is no longer available for a primary specimen, vitreous humor may also be absent at this phase as the globes are notably collapsed. Decompositional fluids collecting in the thoracic cavities become a source of alternative "blood" and other specimens also become more important. The liver and brain are particularly important to collect. Gastric material and urine still may be present and useful again for qualitative analysis.

As decay continues (and especially if insect activity is heavy) fewer and fewer recognizable organs may be available for sampling. An amorphous gray material in the body cavity may be the only substance available. However at this stage, an important specimen may still be available. Specifically the skeletal muscle of the legs, particularly the thighs, should not be overlooked. The quadriceps muscle is particularly resistant to decomposition and may be abundant when no other organs are available. Drugs such as ethanol, cocaine,

and opiate compounds are readily detected from skeletal muscles as well as carbon monoxide which binds tightly to the globin molecule. If mummification or near complete skeletonization has occurred, skin, hair, and fingernails can be collected for toxicologic analysis and after enzymatic breakdown of the keratin. These samples are useful for cocaine, opiate, and heavy metal detection.

Specialized tissues may also be useful for certain analyses. Bone marrow may be valuable for DNA profiling as well as some toxicologic analysis depending on its state. Gastric contents, valuable not only for qualitative identification of ingested drugs, may also be useful for identifying a last meal and potential contamination of food in intentional or unintentional poisoning situations. Blood and urine collected from hospitalized individuals may be able to be located in the blood bank or clinical laboratories. These premortem samples are very useful in forensic toxicology to indicate the state of the individual prior to any therapy and prior to the individual's death. Pharmacokinetic time lines can also potentially be elucidated using these premortem samples. The use of the spleen particularly for detection of carboxyhemoglobin has also been suggested.

In summary, despite the predictable and progressive recycling of blood, body fluid, and the remainder of the human tissues (even in the most toxicologically destitute stages of human decay) any and all body tissues including bone can be considered useful as a forensic toxicologic specimen. Even qualitative results, detecting specific drugs, or classes of drugs such as radioimmunoassay for cocaine, benzoylecgonine, or opiates, may be extremely valuable in assisting the forensic pathologist or death investigator in answering the primary question, "How has this individual interacted with his environment?"

Insects as Toxicological Evidence

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Over the past decade, there has been an increase in drug-related deaths reported in the United States and other countries. In many instances, these deaths are not discovered for a period of several days and, as a result of the decomposition processes, estimations of postmortem intervals are based on entomological data. The data most frequently used are those associated with insect development rates and successional patterns (Catts and Goff 1992; Goff and Flynn 1991; Goff and Odom 1987; Lord *et al.* 1986). Until recently, accuracy of these estimates in deaths involving narcotic intoxication has been subject to question and no studies existed detailing the effects of drugs from decomposing tissues, such as cocaine and heroin, on the rates of development of carrion-feeding insects. Additionally there were few data dealing with effects of other tissue contaminants, such as toxins and environmental pollutants, in decomposing tissues on the rates and/or developmental patterns of insects using such tissues as food sources. Interest has also recently focused on the potential use of carrion-feeding insects as alternate toxicological specimens in situations where more traditional toxicological specimens (such as blood, urine, or solid tissues) are unavailable.

INSECT LIFE CYCLES

Basic to understanding the applications of entomological evidence to toxicological problems is some information concerning the life cycles of the insects. With relatively few exceptions, adult insects lay eggs. The immature forms hatching from these eggs may closely resemble the adult insect, generally lacking only wings and reproductive structures. These immatures (termed nymphs) periodically cast their skins as they increase in size, eventually displaying all of the characteristics of the adult. Most insects of forensic

significance, however, do not hatch into immatures resembling the adults and must pass through three dissimilar stages during their development (egg, larva, and pupa) before reaching the adult stage. None of these developmental stages consume the same food source as the adult insect. The insects most frequently involved in the estimation of the postmortem interval during the first 4 weeks of decomposition are the true flies (or Diptera) and these are also the taxa most frequently used in drug-related analyses. The predominant species encountered are: the Calliphoridae or blow flies and the Sarcophagidae or flesh flies.

Both the Calliphoridae and Sarcophagidae are highly mobile, strong flying species. These flies are typically the first insects to arrive at a corpse. Under tropical conditions, as in the Hawaiian Islands, Calliphoridae may arrive within minutes following death. In more temperate situations, their arrival may require several hours or days, depending on habitat, environmental conditions, and other factors. Adult flies will either begin to lay eggs (oviposit) immediately or feed on the various protein-rich fluids seeping from the corpse and then begin oviposition. On corpses which have not suffered traumatic injury, the initial sites for oviposition will generally be the natural body openings (eyes, ears, nose, mouth, and, if exposed, anus and genitals). Wounds or blood may provide preferential sites for oviposition although this attraction will vary depending on the species of fly involved and the degree of injury.

Blow fly eggs are small (two to three mm), elongate, and white to yellow in color. They are typically laid in large clusters and, during warmer parts of the year, may completely fill natural body openings and wound sites (Figure 1). During cooler periods, where

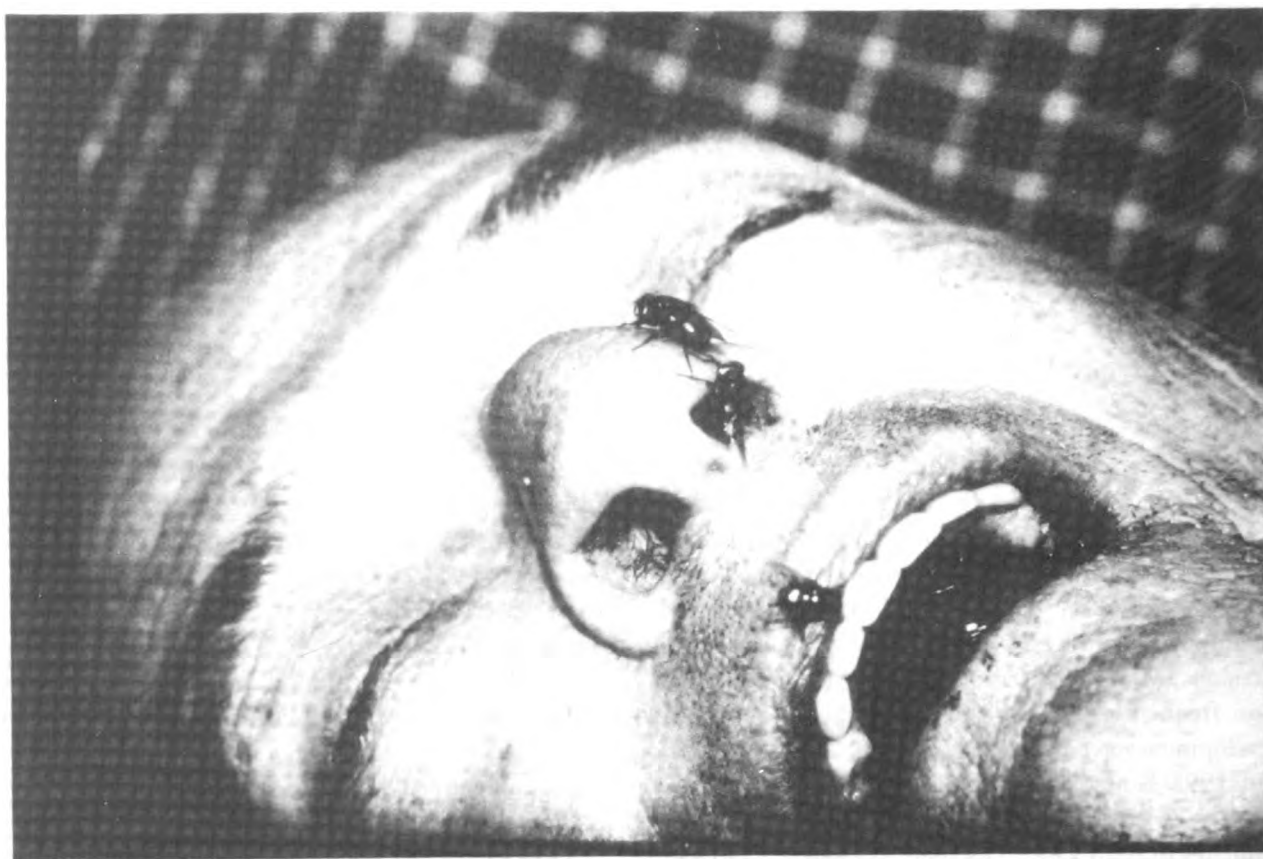


Figure 1. Head of corpse showing eggs of Calliphoridae filling nose.

adult fly populations are smaller, the eggs may be few in number and more difficult to locate, being hidden in cryptic sites such as the inside of the nose or behind the eyelids. Typically the egg stage for blow flies lasts from 1 - 3 days.

When the eggs hatch they produce larvae, commonly referred to as maggots. These are small, somewhat peg-shaped organisms (Figure 2). The anterior end has a pair of mouth hooks which are used both for feeding and in locomotion. The posterior end bears a pair of flattened spiracles through which the maggot breathes (Figure 3). These features, along with size and shape, provide important characters for identification. Maggots grow rapidly, passing through three stages or instars (Figure 2) before reaching full size. The eggs laid on a corpse will typically hatch at the same time, resulting in a mass of maggots which move about the corpse feeding as a group. This group

feeding behavior results in the dissemination of bacteria and production of digestive enzymes which enable the maggots to consume most of the soft tissues of the corpse in a highly efficient manner. Maximum size will be reached in a period varying from several days to weeks, depending on the species involved, numbers of maggots present, and environmental conditions.

After reaching maximum size, maggots undergo a dramatic change in behavior. Feeding ceases and the maggots begin to migrate away from the corpse. This migration is typically to a drier habitat where the maggots burrow into the substrate and begin the process of pupation. During pupation, the outer skin of the maggot becomes hardened and forms a protective cover. This reddish to dark brown pupa somewhat resembles a small football (Figure 4). Within this protective case, the maggot undergoes a reorganization and eventually emerges as an adult fly. The period of time required for

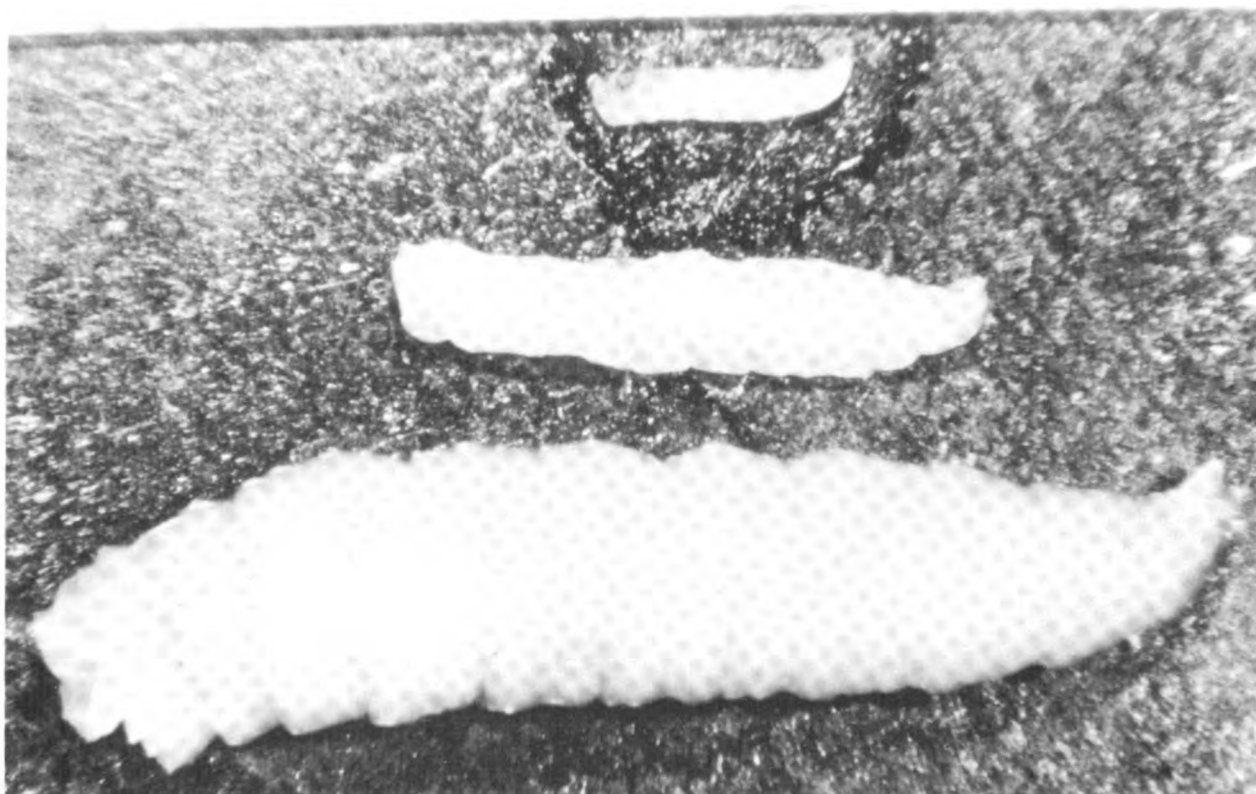


Figure 2. First, second, and third instar larva of Chrysomya Megacephala (Diptera: Calliphoridae).

this transformation varies depending on the species of fly and environmental factors, particularly temperature. The pupal case itself is quite resistant to environmental degradation and may be found in the soil under the corpse for hundreds of years. Blow fly pupal cases can supply valuable forensic information long after the remains have decomposed (Nuorteva 1987).

The life cycle of the Sarcophagidae or flesh flies is quite similar to that of the blow flies, with one important difference. Flesh flies associated with decomposing remains do not lay eggs (oviposit), but rather deposit first instar larvae (larviposition) on remains. This process requires that the developing larvae (maggots) be retained in the body of the female fly longer than for an egg. Thus, the numbers of larvae produced per female are lower for Sarcophagidae than for Calliphoridae. In total numbers of maggots present on the corpse, generally the Sarcophagidae are far outnumbered by the

Calliphoridae. There are exceptions to this in specialized circumstances, as may occur in corpses found indoors (Goff 1991) or exposed to severe environmental conditions.

Careful analyses of the species of flies present on the corpse and their environmental stages combined with a knowledge of fly biology, and local environmental conditions, can provide forensically meaningful information relating to the postmortem interval, movement of the corpse after death from one locality to another, manner of death, and, as will be discussed here, drugs and/or toxins present in the corpse.

DETECTION OF DRUGS AND TOXINS

There are several publications detailing the detection of various toxic and controlled substances through the analyses of arthropods. In these reports,

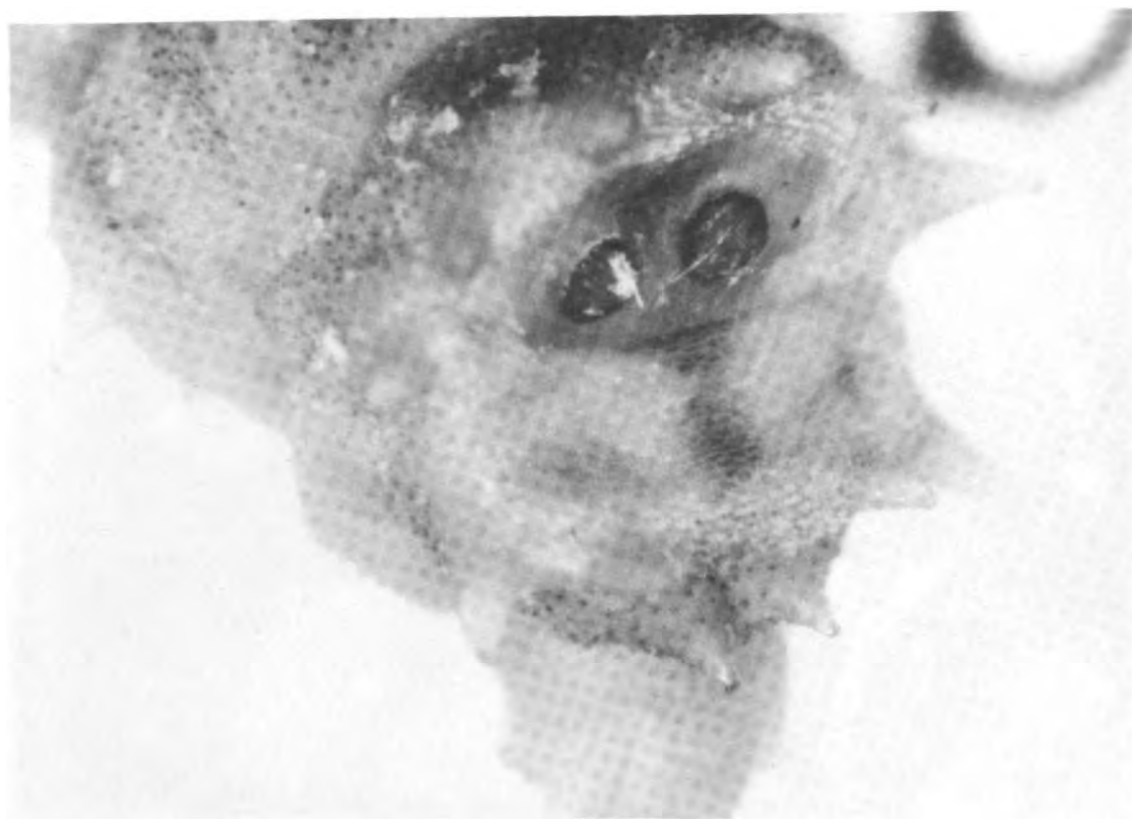


Figure 3. Posterior aspect of third instar larva of *Chrysomya Rufifacies* showing posterior spiracles (Diptera: Calliphoridae).

the arthropods have generally been homogenized and then processed in the same manner as other toxicological tissues or fluids. Analytic procedures have included radioimmunoassay (RIA) techniques, gas chromatography, and thin-layer chromatography.

Nuorteva and Nuorteva (1982) detailed the recovery of mercury from various species of calliphorid maggots fed on fish containing known concentrations of mercury. In this study, it was noted that there was a definite bioaccumulation of mercury by the maggots and this bioaccumulation was related to the presence of mercury in methylated form. In maggots reared on tissues in which 94% of the mercury was methylated, there was a bioaccumulation in which the concentration of mercury in the maggots was 4.3 times greater than in the tissues. In fish containing lesser amounts of methylated mercury, the concentration detected in maggots was only 1.5 times greater than that in tissues.

The mercury ingested by maggots was retained through the pupal stage and detectable in emerging adult flies. Once the adult stage was reached, mercury was rapidly eliminated. After 2 days, the adult flies contained only 50% of the original mercury levels. A more detailed analysis revealed that, for the most part, excretion actually occurred during the pupal stage where accumulated mercury was secreted into the meconium of the hindgut (Nuorteva and Nuorteva 1982). During these studies, no ill effects were generally exhibited by the adult flies or maggots, although some individuals were observed having difficulty in pupation. Maggots which had fed on tissues containing mercury were fed to a staphylinid beetle, *Creophilus maxillosus*, and a secondary bioaccumulation was noted in these predatory beetles. While no ill effects were observed for *C. maxillosus*, subsequent work by Schott and Nuorteva (1983) showed a decrease in activity for the tenebrionid beetle, *Tenebrio molitor*, fed on dried maggots with a

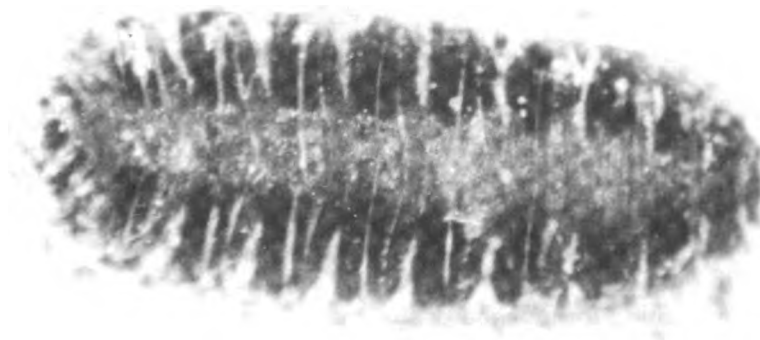


Figure 4. Pupa of *Chrysomya Megacephala* (Diptera: Calliphoridae).

high mercury content. Nuorteva and Nuorteva (1982) had previously noted the occurrence of "Minimata-like symptoms" (consisting of irregularities in motor control and muscular coordination) for adults of that species fed on mercury-containing maggots. No change in life span was noted for the beetles fed on mercury-containing maggots in either study.

In a similar manner, Sohal and Lamb (1977; 1979) demonstrated the accumulation of various metals, including copper, iron, zinc, and calcium, in tissues of adult houseflies, *Musca domestica*. As with the mercury studies cited above, no detrimental effects to the adult flies were associated with the bioaccumulation of these metals.

One example of an application of these types of data was given in a case presented by Nuorteva (1977).

In this instance adult flies reared from a badly decomposed, unidentified female discovered in the rural area of Inkoo, Finland, were analyzed for mercury content in an attempt to determine the geographic origin of the victim. The low mercury content of the adult flies on emergence (0.12 - 0.15 ppm) indicated that the individual came from an area relatively free from mercury pollution. When the individual was identified, she proved to have been a student from the city of Turku, an area relatively free from mercury pollution.

Utsumi (1958) observed that rat carcasses varied in their attractiveness to flies depending on the poison causing death. This research did not, however, include any attempt to detect the toxins in the maggots which eventually developed on the rat carrion. Leclercq and Brahy (1990) noted the detection of arsenic poisoning through the analyses of species of Piophilidae, *Fannia*,

and Psychodidae in a case from France.

Detection of an organophosphate insecticide, Malathion, was reported by Gunatilake and Goff (1989). In this instance, a 58-year-old male with a previous history of suicide attempts was discovered in the crawl-space under his mother's home in Honolulu. Adjacent to the remains was a bottle of Malathion-50 with approximately 177 ml missing. Both tissues and maggots of two species of Calliphoridae were analyzed for Malathion using gas chromatography. The fat tissues of the corpse had Malathion detected at 17 mg/kg. The combined sample of maggots of *Chrysomya megacephala* and *Chrysomya rufifacies* showed Malathion at a concentration of 2,050 ug/g. It is of significance that the developmental stages of both species of *Chrysomya* in this case were indicative of a postmortem interval of 5 days. The individual had last been seen alive 8 days prior to the discovery of the corpse. It is possible that the presence of Malathion in the tissues of the corpse may have served to delay oviposition for a period of several days. This is supported by the presence of only two species of flies on the corpse. In outdoor situations in the Hawaiian Islands, a far greater number of insect species should have been present on the corpse at either a 5 or 8-day interval. Typically, other species of flies and predatory Coleoptera in the families Staphylinidae and Histeridae would have been expected to have invaded the corpse and the surrounding substrate.

Beyer *et al.* (1980) detailed the case of a 22-year-old female whose decomposed remains were discovered 14 days after she had last been seen alive. The individual had a previous history of five suicide attempts. An empty bottle from a prescription for 100 tablets of phenobarbital, filled 2 days before she was last seen alive, was found in a purse adjacent to the remains. As there were no tissues on the almost completely skeletonized body available for analysis, larvae of the calliphorid, *Cochliomyia macellaria*, feeding on the remains, were collected and analyzed for drug content. Phenobarbital was detected by gas chromatography and confirmed by thin-layer chromatography at a concentration of 100 ug/g.

Kintz *et al.* (1990) have detailed further detections of prescription drugs through analyses of maggots. In one case, the subject was a male with a determined postmortem interval of 67 days. There were identifiable organs present for toxicologic analyses using liquid chromatography. These included heart, liver, lung,

spleen, and kidney, in addition to bile. Calliphorid maggots were also present and analyzed. Results of analyses showed five drugs present in the maggots as well as in the bile, heart, liver, and lung (triazolam, oxazepam, phenobarbital, alimemazine, and clomipramine). Triazolam was not detected in the spleen and kidney, although other drugs were present. In this case, it was not possible to establish any correlations between the concentrations of drugs in the maggots and the human tissues. It is of interest to note that Kintz *et al.* (1990) observed fewer endogenous peaks in chromatograms obtained from maggot extractions than those from human tissues. Further evidence of the toxicological potentials of insect larvae was provided by Kintz *et al.* (1990) through their detections of other drugs (bromazepam and levomepromizine) in maggots obtained from human remains. More recently, Wohlenberg *et al.* (1992) reported during the meetings of the American Academy of Forensic Sciences in New Orleans that they were able to detect nortriptyline from maggots infesting the nearly skeletonized remains of a 40-year-old male using gas chromatography mass spectrography. In a similar manner, Goff *et al.* (in press) demonstrated amitriptyline in maggots fed on rabbit tissues containing the drug. They were further able to demonstrate this drug through analyses of the empty puparia.

Recent studies by Introna *et al.* (1990) detailed the results of analyses of maggots fed on liver tissues from 40 cases in which opiates had been detected during the postmortem exam. In this study, opiates were effectively detected through analyses of maggots which had fed on opiate contaminated tissues using RIA techniques. A significant correlation between the concentrations of opiates in the liver tissues and the paired maggots sampled was also observed. While the qualitative relationship was quite clear from their results, the quantitative relationship was less clear and further research in this area is suggested. Similar results were presented for qualitative detection by Goff *et al.* (1989; 1991) for studies using known dosages of cocaine and heroin administered to rabbits.

Lord (1991) presents an account of the use of toxicological analyses of maggots to determine cocaine intoxication. In that case, the almost completely skeletonized remains of a 29-year-old male intravenous drug user were discovered in February, almost 5 months after he had last been seen alive. His girlfriend indicated that she believed he had used intravenous cocaine

immediately prior to his disappearance. Associated with the remains were numerous maggots and pupae. Skeletal muscle along with maggots were submitted for toxicological analysis using gas chromatography. Both tissues and maggots tested positive for cocaine and benzoylecgonine. As in previous cases, the sample from the maggots had less interference from by-products of decomposition and provided more suitable samples for quantitation. The empty puparial cases were subsequently tested and found to be weakly positive for cocaine. It appears that cocaine and/or benzoylecgonine are deposited in the protein matrix of the pupal cases and, thus, may be detectable for years following death. The extraction method used in this case was similar to that for human hair (Baumgartner *et al.* 1989; Graham *et al.* 1989). In a somewhat similar type of situation, Manhoff *et al.* (1991) were able to detect cocaine at a concentration of 0.090 mg/L using beetle feces and also calliphorid larvae collected from decomposed human remains.

EFFECTS OF DRUGS IN TISSUES ON DEVELOPMENT OF INSECTS

While the previously cited studies have documented the potential of maggots as alternate specimens for toxicological analyses, they have not addressed questions concerning the effects of these substances on the rate of insect development. In the estimation of the postmortem interval, particularly during the first 2 - 4 weeks of decomposition, it is assumed that the insects, particularly the Diptera, will develop and known, predictable rates for given climatic conditions. That this might not always be the case when drugs are involved was first demonstrated by Goff *et al.* (1989) during their studies on the effects of cocaine in decomposing tissues on the rate of development of the sarcophagid *Boettcherisca peregrina*. In this study, domestic rabbits were administered dosages calculated by weight to represent 0.5, 1.0, and 2.0 times median lethal dosages of cocaine. Those animals receiving the

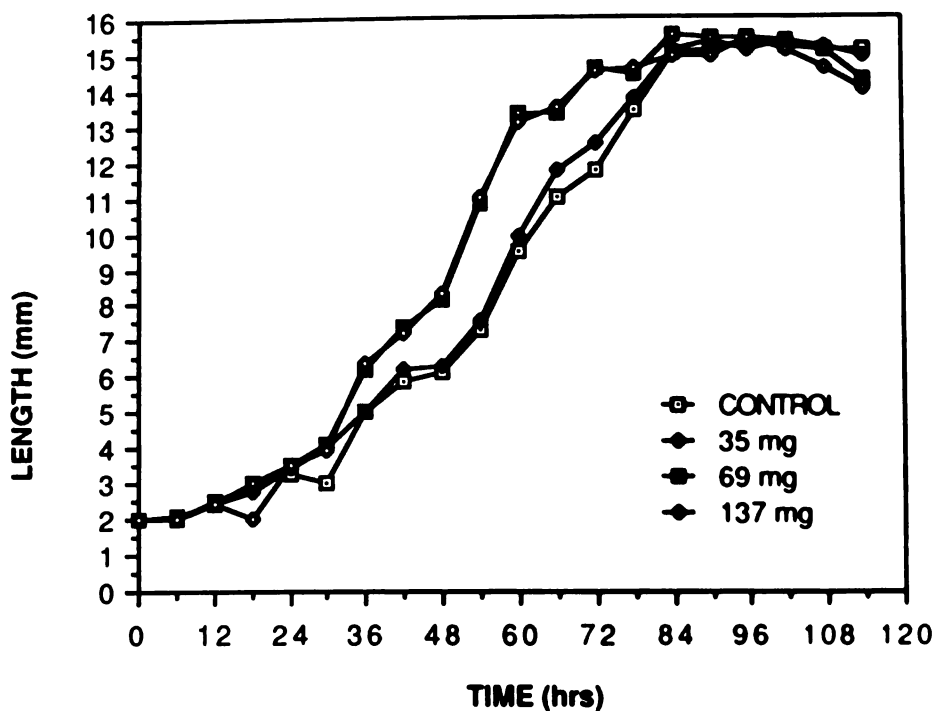


Figure 5. Development rates of *Boettcherisca peregrina* maggots fed on rabbit liver tissues containing varying amounts of cocaine. Reproduced from Goff *et al.* (1989) with permission of the Entomological Society of America.

Table 1. Pupal duration and adult emergence for colonies of *B. Peregrina* reared on rabbit liver tissue containing varying amounts of cocaine. Reproduced from Goff *et al.* (1989) with permission of the Entomological Society of America.

Colony	Total Pupae Obtained	Total Adults Emerging	Percent Emerging	X Duration in Hours of Pupal Stage (Range)
Control	459	416	91	255 (234 - 312)
35 mg	445	407	91	253 (234 - 300)
69 mg	390	359	92	261 (240 - 294)
137 mg	708	649	91	252 (228 - 294)

lethal dosages were allowed to expire as a result of the action of the drug and the animal receiving the sublethal dosage and control were sacrificed in a CO₂ chamber. Presence of cocaine and/or its major metabolite benzoylecognine were confirmed by analyses of samples of liver tissues. Livers and spleens were removed from animals following death and exposed to a colony of *Boettcherisca peregrina* for larviposition. Samples were measured from each colony at 6-hour intervals and samples of maggots removed at 24-hour intervals for drug analyses. Samples of maggots from all colonies feeding on tissues containing cocaine and/or benzoylecognine were positive for the drug or metabolite. Control liver and maggots were negative for both substances. In this study, there were two patterns of development detected. The control and sublethal colonies developed at approximately the same rate, as indicated by the total length of the larvae measured. By contrast, beginning at hour 36, the 1.0 and 2.0 times median lethal dosage colonies developed more rapidly (Figure 5). There were significant differences detected ($P < 0.05$) until hour 76. Pupation was first observed in the twice median lethal dosage colony, followed by the median lethal, sublethal, and control colonies. No significant variation in length of the pupal period was observed (Table 1) and no differences in pupal mortality were detected. Adults from all colonies produced viable offspring when supplied with liver 13 days following eclosion of adults. The colonies were followed for four generations and no abnormalities were observed.

Similar studies were conducted by Goff *et al.* (1991) for maggots feeding on decomposing tissues containing heroin. As above, dosages were calculated

based on weight to represent 0.5, 0.75, 1.00, and 2.00 times median lethal dosage. Rates of development, as indicated by total lengths of maggots, were not significantly different ($P > 0.05$) until hour 18. At that point, maggots in all colonies fed on tissues containing heroin began to develop at rates significantly greater than those observed in the control colony. These differences (Figures 6 and 7) continued through hour 96, when maximum larval lengths were recorded for maggots in all colonies. Maggots from all colonies fed on tissues containing heroin (as morphine) were significantly larger than maggots from the control colony ($P < 0.05$). Duration of the pupal period was significantly longer for maggots from colonies fed on tissues containing heroin and was directly proportional to the concentration of this drug (as morphine) in the tissues (Table 2). This study has indicated that an estimate of the postmortem interval based on normal development of *B. peregrina* could have an error of up to 29 hours, if based on the larval stage, and 18 - 38 hours, if based on the pupal stage.

Studies conducted by Goff *et al.* (1992) on the effects of methamphetamine on the development of another species of sarcophagid fly, *Parasarcophaga ruficornis*, exhibited some similarities to the above studies on cocaine and heroin, but also some significant differences. These studies, although somewhat preliminary in nature, show similar increases for colonies fed on decomposing tissues containing the 1.0 and 2.0 median lethal dosages of methamphetamine from hour 24 through hour 60, while the sublethal dosage and control colonies developed at approximately the same rate. Following hour 60, the rate of growth for the 1.0

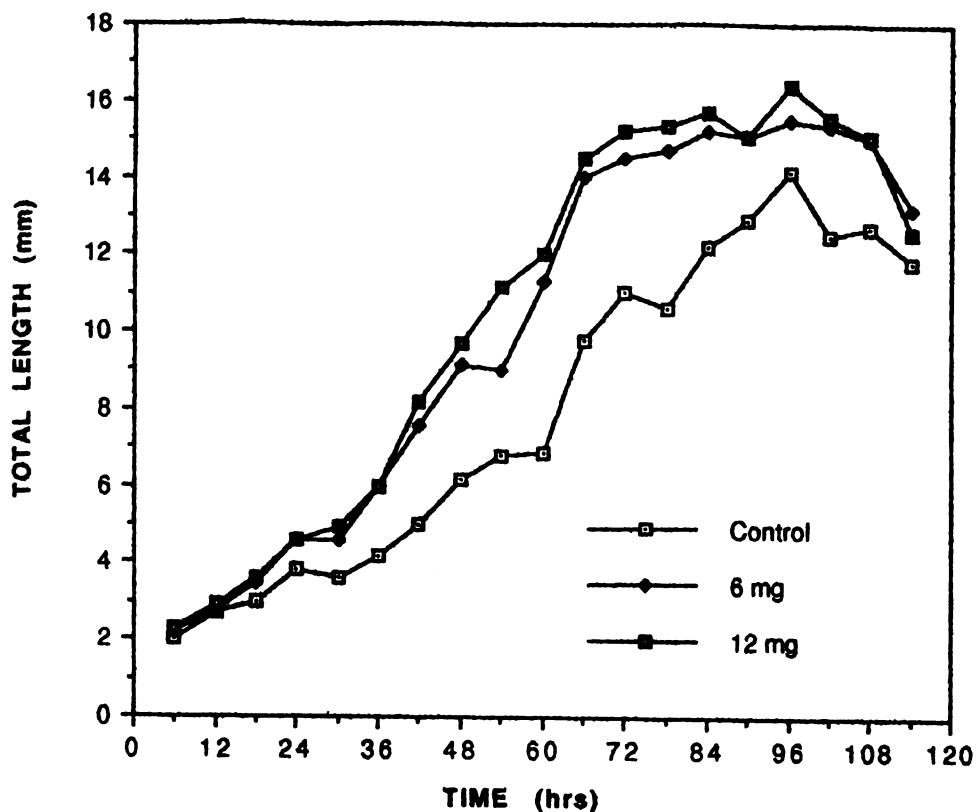


Figure 6. Development rates of *Boettcherisca Peregrina* maggots fed on rabbit liver tissues containing varying amounts of heroin. Reprinted with permission from Goff et al. (1991) copyright ASTM.

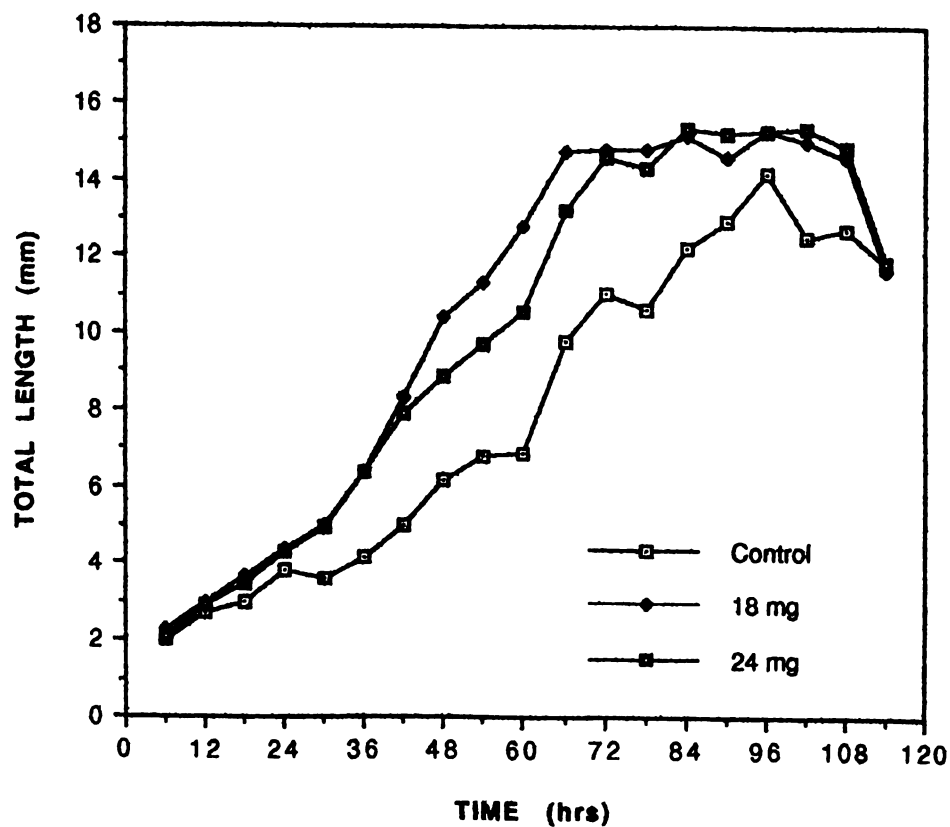


Figure 7. Development rates of *Boettcherisca Peregrina* maggots fed on rabbit liver tissues containing varying amounts of heroin. Reprinted with permission from Goff et al. (1991) copyright ASTM.

Table 2. Adult emergence, pupal duration, and total developmental period for colonies of *Boettcherisca Peregrina* reared on rabbit liver tissue containing varying amounts of heroin.* Reproduced from Goff *et al.* (1991), copyright ASTM (reprinted with permission).

Colony	Total Pupae Obtained	Percent Emerging	X Duration in Hours of Pupal Stage (Range)	X Total Development Period (Range)
Control	451	89	253 (246 - 276)a	397 (378 - 432)a
6 mg	209	95	271 (254 - 282)b	406 (386 - 450)b
12 mg	150	88	279 (272 - 282)c	422 (404 - 450)c
18 mg	338	97	273 (272 - 312)bc	411 (404 - 456)bc
24 mg	310	89	291 (288 - 194)c	439 (408 - 450)c
* Figures in a column followed by the same letters are not significantly different.				

median lethal dosage colony slowed, and only the 2.0 median lethal colony continued to develop at a rate significantly greater than the other colonies ($P < 0.05$). The maximum sizes of maggots were reached earlier in colonies fed on tissues from animals receiving lethal and twice lethal median dosages of the drug than in the control and sublethal dosage colonies (Table 3). The greatest total length was attained by the control colony maggots (Table 3). Unlike the situation for heroin, there were no significant differences in duration of the pupal stage for maggots fed on tissues containing methamphetamine, although all these colonies had

durations of the pupal stage which were significantly shorter than for the control colony. Pupal mortality rates for the drug-treated colonies appeared to be inversely proportional to the dosage of methamphetamine administered to the animal model, with the pupal mortality observed for the control colony and 2.0 median lethal dosage colony approximately equal (Table 3). Unlike the situation for both cocaine and heroin, there were effects observed in the reproduction in the 1.0 and 2.0 median lethal dosage colonies. The 1.0 median lethal dosage colony failed to reproduce during the first generation and the 2.0 median

Table 3. Maximum larval lengths, minimum durations of developmental stages, and puparial mortality for *Parasarcophaga Ruficornis* reared at 26°C on rabbit liver tissues containing varying amounts of methamphetamine*. Reproduced from Goff *et al.* (1992), copyright ASTM (reprinted with permission).

Colony	Maximum Length of Larva (mm)/Time Maximum Observed (h)	Minimum Duration of Larval Stage (h)	Minimum Duration of Pupal Stage (h)	Percent Pupal Mortality
Control	20/90	138	338a	4.4a
37.5 mg	18/90	132	294b	60.6b
71.4 mg	17/78	114	295b	21.4c
142.9 mg	18/78	138	290b	5.5a

* Figures in a column followed by the same letters are not significantly different ($P > 0.05$).

Table 4. Durations of larval stage; larval mortality; puparial weights, lengths, durations, and mortalities for colonies of *Parasarcophaga Ruficornis* reared on rabbit liver tissues containing varying amounts of amitriptyline.

Colony	X Duration of Larval Stage (Hours) ¹	Larval Mortality	X Pupal Weight (g)	X Pupal Length (mm)	X Duration of Pupal Stage (Hours)	Pupal Mortality
Control	159.9a (N = 189)	5.5%	1.01a (N = 189)	10.78a (N = 20)	313.8 (N = 185)	4.2%
300 mg	173.4b (N = 85)	57.5%	1.06a (N = 85)	10.85ab (N = 20)	311.8 (N = 82)	3.5%
600 mg	190.1c (N = 119)	40.5%	1.15b (N = 119)	11.28b (N = 20)	361.1 (N = 111)	5.0%
1000 mg	172.7b (N = 115)	42.5%	1.14b (N = 115)	11.35b (N = 20)	348.2 (N = 115)	0.0%
¹ Figures in a column followed by the same letters are not significantly different (P > 0.05).						

lethal dosage colony failed during the second generation. In both cases nonviable larvae or eggs were produced.

In recent studies concerning the effects of the tricyclic antidepressant amitriptyline on the developmental patterns of *Parasarcophaga ruficornis*, Goff *et al.* (in press), no significant differences were observed in the rates of development for colonies reared on decomposing tissues from animals receiving 0.5, 1.0, and 2.0 times median lethal dosages of amitriptyline and the control. The larval mortality rates for lethal and twice lethal colonies were significantly greater than for the control. Sizes of puparia, as indicated by both length and weight, were significantly greater (weight and length) for pupae from colonies reared on tissues from lethal and twice lethal dosages of the drug. Larval mortality rates were significantly higher ($P < 0.05$) for colonies reared on tissues from animals receiving the drug than for the control colony (Table 4). There were no significant differences in mortality during the pupal stage between colonies, although the duration of the pupal stage was significantly longer for the lethal and twice lethal colonies than for the sublethal and control (Table 4).

While there have been, to date, few applications of these data to actual cases, the case detailed by Lord (1991) illustrates the potential significance of these alterations in the rates of larval and pupal development.

The corpse of a Caucasian female approximately 20 years old, was discovered in pine woods area northeast of Spokane. The corpse was physically in the early bloat stage of decomposition, with an extensive population of maggots on the face and upper torso. Maggots were submitted after 5 days of refrigeration and reared to the adult stage on beef kidney. There were two species present: *Cynomyopsis cadaverina* and *Phaenicia sericata*. *Phaenicia sericata* typically oviposits within 24 hours following death and *C. cadaverina* oviposits 1 - 2 days following death. There were three classes of maggots present, based on total length. The first consisted of maggots measuring 6 - 9 mm in length and consistent with an interval of 7 days. The second consisted of smaller maggots, indicative of multiple broods under development. The third class consisted of a single maggot from the nasopharyngeal area, measuring 17.7 mm in total length and indicating a time period of approximately 3 weeks. This time period was not possible, given the other data associated with this corpse. The possibility that the maggot had migrated from another nearby source was eliminated, as no such carrion were located by investigators and the probability that more than one maggot of that size would have been found. An alternate explanation was that the maggot's growth had been enhanced in a particular pocket in the nasal region containing a concentration of cocaine. A subsequent investigation showed the victim had a history of cocaine abuse and a witness later testified that the

victim had snorted cocaine shortly before her death.

CONCLUSIONS

Insects and other arthropods can prove to be valuable tools in investigations of homicide, suicide, or other unattended deaths. In addition to the recognized applications to estimation of postmortem intervals, insects may serve as reliable alternate specimens for toxicological analyses in the absence of tissues and fluids normally taken for such purposes. In cases of decomposed remains, analyses of insects may give more reliable qualitative analyses than the decomposed tissues. While the data presented above concerning the effects of drugs and metabolites in tissues on the development rates of Diptera larvae are limited in scope, it is not unreasonable to assume that other substances in tissues may also produce changes in the rates and/or patterns of development. Until appropriate baseline data are available, care must be taken in the interpretations of arthropod developmental patterns in cases where drugs and toxins may be a factor. In addition, it becomes essential that the forensic entomologist be made aware of any data indicating the presence of these substances in the remains. This will allow for needed corrections of the life cycle data, based on the substances involved, and result in more accurate interpretations.

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Wildlife Forensic Toxicology: A Case Study of Fluorescent Taggant in Elk

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Trade in live elk is profitable; game ranches across North America have increased the value of an average elk to \$5,000. A record bull will bring about \$15,000.

The Chama Land and Cattle Company operates a 32,000-acre game ranch in New Mexico where wild animals are managed for hunting purposes under seminatural conditions. The ranch is surrounded by a 22-mile, 8-foot high, game-proof fence that encloses privately owned elk and sits in the migration path of a herd of wild elk. There are gates along the fence, including four in a remote quarter-mile stretch on the northeast section.

New Mexico state game officials and U.S. Fish and Wildlife Service agents suspected that the gates around the park were being opened at periodic intervals to allow wild elk to enter the game ranch. Bales of hay, to which large numbers of elk were attracted, were regularly set outside the gates. On January 18, 1989, a state district judge in Santa Fe, New Mexico, approved a sealed court order allowing wildlife agents to enter the Chama Land and Cattle Company and covertly sprinkle ultraviolet tracking powder on hay outside the game ranch near the gates along the fence in the northeast section. The expectation was that the fluorescent powder would adhere to the chest, legs, and mouth of elk feeding on the hay. On February 9, 1989, special agents of the U.S. Fish and Wildlife Service reentered the ranch and found ultraviolet powder on snow-covered ground within the private game park.

On February 18, 1989, employees of the Chama Land and Cattle Company loaded 109 elk, which were sold to a game ranch in Canada, from corrals inside the ranch onto 2 aluminum double-deck livestock trailers. En route to Canada, wildlife agents detained and seized the elk in Colorado under authority of the Lacey Act

(which prohibits interstate traffic in illegal wildlife and wildlife parts).

When field agents examined the elk for signs of the fluorescing powder they discovered that none of the elk showed fluorescence in the expected areas, but that the urethra and anus of these animals fluoresced bright blue. It became clear that the elk had eaten the fluorescent powder. Field agents proceeded to collect 98 urine samples from the elk and tissue samples from 11 elk that had died in transport.

Initially it was expected that the presence of an externally deposited fluorescing powder would indicate a captured wild elk, but the issue became more complex when the elk consumed the powder because it was noted that some elk have inherent urine fluorescence. Six months after the collection of the evidence, the National Fish and Wildlife Forensics Laboratory was contacted and asked to characterize the fluorescent powder and the biological samples to determine commonality of source.

MATERIALS AND METHODS

Fluorescent powder samples were solubilized in HPLC grade acetone and filtered. Ten grams of elk kidney were homogenized and one gram was transferred to a screw cap centrifuge tube. Two milliliters of methanol (Fisher Scientific) were added to the ground tissue and rotated for 4 hours. Homogenates were centrifuged at 2,500 RPM for 5 minutes and 1 ml of supernatant was transferred to a disposable C18 reverse phase solid extraction column. Evidence samples were added directly to the C18 solid phase extraction column. Reverse phase C18 extraction columns were then placed in the solid phase vacuum extraction unit (PrepTorr) and washed. Evidence samples were added, rinsed with water, and eluted twice with 150 μ L of methanol

followed by 100 uL of HPLC grade acetone (Fisher Scientific). β -Glucuronidase (β -glucuronoside glucuronosohydrolase; EC 3.2.1.31), sodium chloride, sodium acetate, phenolphthalein glucuronidate were purchased from Sigma for determining glucuronic acid conjugation. The assay was conducted as described by Fishman *et al.* (1948).

All evidence samples were analyzed in a Hewlett Packard 5988A Gas Chromatograph/Mass Spectrometer (GC/MS) system via the automated liquid sampler. Separation was obtained using a 30-meter DB-5 capillary column with a film thickness of 0.25 μ m (J & W Scientific). Instrumental conditions were as follows: injector temperature-150°C; interface temperature-300°C; source temperature-200°C; carrier gas was helium at 15 ml/min.; oven temperature-100°C ramped to 200°C at 10° per minute. Reference compounds (Sigma) were characterized by their mass spectra and eluted as follows:

Compound	Elution Time (minutes)
4-methyl-phenol	3.6
diphenylamine (internal standard)	13.9
2-methyl-benzenesulfonamide	14.6
4-methyl-benzenesulfonamide	17.1
7-(diethylamino)-4-methyl-coumarin	19.5

CHARACTERIZATION OF THE FLUORESCENT POWDER

The fluorescent powder had been purchased from Radiant Company under the trade name of Clear Resinz-1029® and is a polymer meant to be used in paint. Radiant Company, when manufacturing Clear Resinz-1029® uses, in part, a Mobay Corporation product with the trade name "Blankophore-SOL®." Patent rights did not allow Radiant Company or Mobay Corporation to share the chemical identities of the powders. GC/MS chemical analysis revealed Blankophore-SOL® to be 7-(diethylamino)-4-methyl-coumarin. Analysis of the monomeric constituents of Clear Resinz-1029® consisted of:

4-methyl-benzenesulfonamide	79.8%
7-(diethylamino)-4-methyl-coumarin	12.6%
2-methyl-benzenesulfonamide	4.5%
unknown contaminants	3.1%

Strong fluorescence of Clear Resinz-1029® was observed at 10 ug/ml with a long wave ultraviolet lamp, but the level of concentration was below detectable limits of the GC/MS. The ultraviolet fluorescence is caused by 7-(diethylamino)-4-methyl-coumarin. The function of the 4-methyl-benzenesulfonamide appears to be that of a special purpose additive.

Since 4-methyl-benzenesulfonamide was the principal ingredient detected in the fluorescent powder, it was speculated that the metabolites that could be produced by oxidation of this compound when catalyzed by cytochrome P-450 monooxygenases were: (a) aromatic hydroxylation products to form mono or dihydroxy-4-methyl-benzenesulfonamide; (b) desulfuration products to form toluene; (c) amide hydrolysis to form 4-methyl-benzenesulfonic acid (Neal 1975); or (d) oxidation of the methyl group to form 4-carboxy-benzenesulfonamide (Ball *et al.* 1977a; 1977b). These reactions are illustrated in Figure 1. Some or all of these metabolic products were expected in the suspect animals if they had ingested Clear Resinz-1029®, the fluorescent powder.

ABBREVIATED ELK FEEDING STUDY

Some elk have inherent fluorescence in their urine, probably due to naturally occurring fluorescent substances such as proteins (due to the presence of the amino acids tyrosine and tryptophan), vitamins A and E, riboflavin, purines (nucleotides), chlorophyll, porphyrins, and coumarin (*e.g.*, sweet clover). Law enforcement agents captured four elk in a distant location (Craig, Colorado) and collected their urine to use as a negative control. Two of these animals were subsequently fed the fluorescent powder and urine samples were collected over a period of 3 consecutive days to use as a positive control.

Fluorescence experiments carried out on a Perkin-Elmer Fluorimeter indicated a unique spectrum for the fluorescent powder, but the spectral pattern was not found in the positive controls. Negative controls could not be distinguished from positive controls, presumably due to interfering urine compounds. Visual examination with a long wave ultraviolet lamp showed the positive

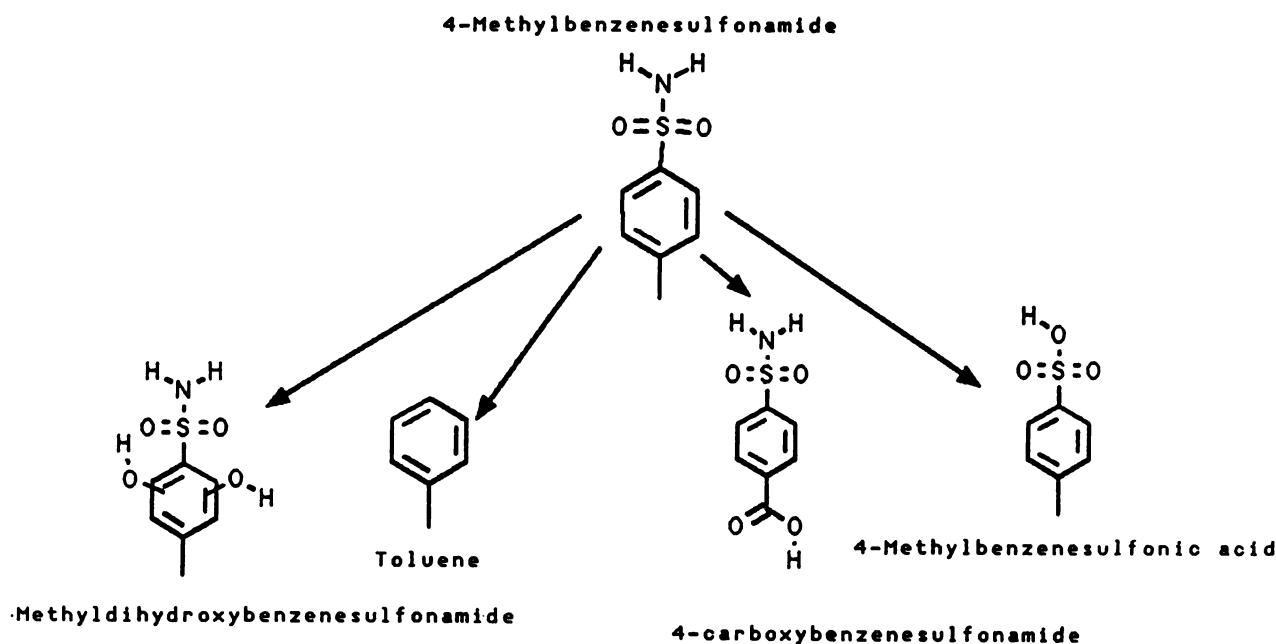


Figure 1. Possible metabolites from the oxidation and amide hydrolysis of 4-methylphenol.

with a long wave ultraviolet lamp showed the positive controls to have a characteristic blue-white fluorescence and the negative controls to have a yellowish fluorescence.

Positive and negative controls were then analyzed by GC/MS. None of the compounds found in Clear Resinz-1029® were detected, but 4-methylphenol was present in the positive controls. The two positive controls were treated with β -glucuronidase for the presence of conjugated parent compounds, but none were detected. In the presence of β -glucuronidase, the concentration of 4-methylphenol increased by 27.8% suggesting that 4-methylphenol might be excreted both free and conjugated with glucuronic acid.

The principal distinction between the positive and negative controls was the quantitative difference in the levels of 4-methylphenol. Analysis of the negative controls did not detect 4-methylphenol whereas analysis of the positive controls revealed an average concentration of 116 ug/ml.

ANALYSIS OF KIDNEYS AND URINE

Eleven elk died during transport and seizure operations and were necropsied in the field. Kidney samples were submitted for GC/MS chemical analysis;

one elk kidney sample contained 4-methylbenzenesulfonamide and the other ten contained 4-methylphenol.

These data were compared to those resulting from the analysis of urine collected from 25 elk in Yellowstone National Park and 12 elk from elsewhere in Wyoming. Table 1 shows the quantitative differences of 4-methylphenol among the Yellowstone elk, Wyoming elk, and the kidneys of the 11 New Mexico elk carcasses suspected of ingesting the fluorescent powder.

The average concentration of 4-methylphenol in the seized elk kidney samples was 115.5 ug/ml. The average concentration of 4-methylphenol in urine samples from Yellowstone National Park was 2.7 ug/ml; the Wyoming elk urine concentration was 69.4 ug/ml. The New Mexico data and the Yellowstone urine data were compared by *t*-test statistic with $p < 0.05$ considered significant. The comparison shows that the quantitative difference is statistically significant ($t = 4.10$). This result must be interpreted cautiously, however, since it is not certain how similar elk kidney homogenate samples are with elk urine. Conversely, when the elk kidney data and the Wyoming elk urine were compared by *t*-test statistic with $p < 0.05$ considered significant, the comparison showed that the quantitative difference is not statistically significant ($t = 0.84$).

Table 1. Concentration of 4-methylphenol (ug/ml) in the elk kidney samples from New Mexico and the elk urine samples from Yellowstone National Park and elsewhere in Wyoming.			
	Chama Land and Cattle Company Kidney Samples	Yellowstone National Park Urine Samples	Wyoming Urine Samples
Average (ug/ml)	115.5	2.7	69.4
Median (ug/ml)	75.0	0	36.6
Standard Error	44.2	1.9	33.1
Range	0 - 438	0 - 48	0 - 407
Sample Size	11	25	12

Since 4-methylphenol is detected in animals never exposed to the fluorescent powder, there must be a precursor or natural source of 4-methylphenol in elk feed. Urine samples in Yellowstone National Park were collected in the winter months and the Wyoming samples were collected in the summer months. Summer offers a wide range of food sources that are not available during the winter. Since the New Mexico elk were captured and seized in the winter the comparison, in this instance, with the Yellowstone elk might be

appropriate.

Since the feeding study indicated that elk which had no 4-methylphenol did exhibit it in their urine after the ingestion of Clear Resinz-1029® and since the comparison of elk kidneys with the Yellowstone population is significant, it may be cautiously suggested that the biotransformation of 4-methylbenzenesulfonamide in elk involves desulfuration with subsequent hydroxylation (Figure 2).

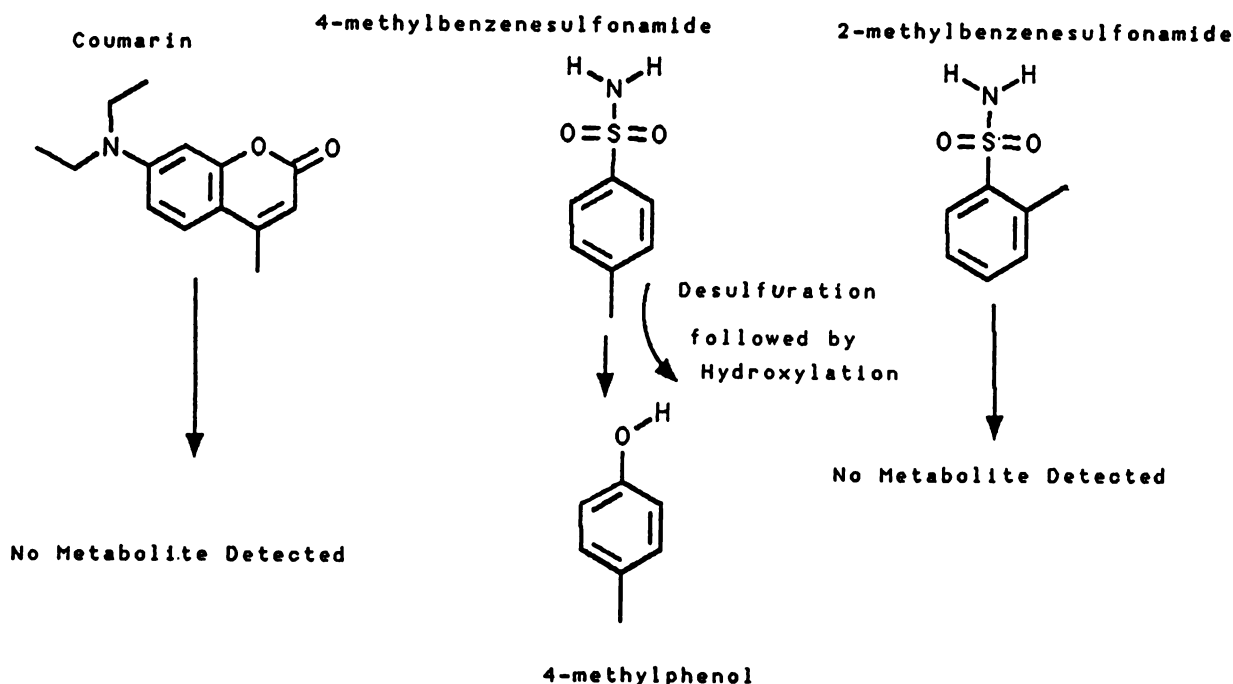


Figure 2. Principal components detected in Clear Resinz-1029 and the suggested biotransformation of 4-methylbenzenesulfonamide to 4-methylphenol via desulfuration with subsequent hydroxylation.

urethra swabs revealed various amounts of 4-methylphenol which were not statistically significant. It was later discovered that the evidence urine samples had been collected 10 days after the last possible exposure to the fluorescent powder. This may account for the low levels of 4-methylphenol since Ball *et al.* (1977b) has shown that rats fed 4-methyl-benzenesulfonamide metabolized and excreted 80% of the dose within 24 hours.

Analysis of debris (hay and feces) from the transport truck detected the presence of 4-methyl-benzenesulfonamide. Nighttime search inside the Chama Land and Cattle Company property detected fluorescing spots in the snow. Three samples of snow were submitted for analysis and all samples contained 2-methyl-benzenesulfonamide, 4-methyl-benzenesulfonamide, and 7-(diethylamino)-4-methyl-coumarin, indicating that the fluorescent spots were consistent with Clear Resinz-1029®.

CONCLUSIONS

The predicted metabolic products outlined in Figure 1 were not found, but 4-methylphenol was detected in many of the evidence samples. 4-Methyl-benzenesulfonamide (toluene-4-sulfonamide) has been known to undergo oxidation to 4-carboxy-benzenesulfonamide (4-sulfamoyl-benzoic acid) in dogs and rats, and is a well-documented route of metabolism of methylbenzenes (Williams 1959). In elk 4-methyl-benzenesulfonamide may undergo desulfuration with subsequent hydroxylation to produce both free and conjugated 4-methylphenol.

The biotransformation of 4-methylbenzenesulfonamide to 4-methylphenol in elk may be due to the fact that ruminants have a digestive strategy different from caecalids (nonruminants); ruminants ferment cellulose and degrade toxins in the rumen whereas nonruminants use their hind gut (Guthrie 1990).

The presence of 4-methyl-benzenesulfonamide in one elk kidney sample suggested that some wild elk had been exposed to the fluorescent powder deposited outside the game ranch boundaries. The presence of the fluorescent powder on the snow outside the game ranch suggested transfer into the corrals over a distance of two miles.

In this case toxicological studies of the fluorescent powder were not conducted since it was expected that the presence or absence of the taggant would suffice to demonstrate that elk were being taken from the wild. It also was infeasible to conduct a controlled toxicological assessment of Clear Resinz-1029® after the fact. Future use of chemical taggants will involve toxicological evaluation from its inception for the purpose of the parent and metabolic compounds in ruminants.

ACKNOWLEDGMENTS

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Murder By Poison

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Murder by poison is probably as old as mankind. Early examples include hemlock, a nicotinic-like drug used by the Greeks; and aconite, a cardio-pulmonary depressant used by the ancient Chinese (Casarett and Doull 1975).

Toxicology actually owes its inception to the use of poison as a murder weapon, when, in the 1840s, Matthieu J. B. Orfila, the father of modern toxicology, detected arsenic in the tissues of the murder victims of Marie Lafarge (Casarett and Doull 1975). Subsequently, the main thrust in the development of forensic toxicology was the detection of successively more sophisticated toxins: the actions of strychnine and curare by Bernard in 1850, and the separation of alkaloids by Stas-Otto in 1851. The field later expanded to include the development of antidotes, the first being dimercaprol which counteracted the arsenic-containing war gases of the 1939-1945 conflict (Casarett and Doull 1975).

In recent years, the field of forensic toxicology has, unfortunately, begun to change from a science that is primarily concerned with the isolation, detection, and identification of poisons associated with deliberate or accidental ingestion, to a science now consumed with the documentation of the exposure to drugs of abuse. Today, few forensic toxicology laboratories routinely test for heavy metals, while others test only for a limited number of compounds, mostly drugs. Some laboratories are so specialized that they only test for five drug groups (cocaine-related alkaloids, opiates, amphetamines, phencyclidines, and cannabinoids) and then only report results for drugs found above preset cutoff concentrations, reporting any other findings as negative.

The discussion below is crafted to emphasize that murders by poison, although only a small portion of the total number of homicides, nonetheless occur regularly. And further, that current forensic toxicology

analytical protocols, with their emphasis on drugs of abuse, may tend to miss many poisonings even when classical toxins (*e.g.*, cyanide or arsenic), are used. The cases presented herein are arranged by motive rather than by the analytical method applied or the classification of the toxin, because the motives for murder are more finite than the number of potentially lethal chemical agents. Of the motives presented, some are classical such as revenge, monetary gain, and professional jealousy; while others are more unusual and less obvious, for example, accidental poisoning during the commission of a felony or some sort of terrorist activity.

REVENGE

Revenge is probably one of the most common and the most basic motivation for murder. It is perceived by some to be a natural human response to the feeling that one has been wronged. The first case presented is a classic—it involves the attempted poisonings of a federal judge, a prosecutor, and an arresting officer.

The attempted multiple-murder scenario begins with the wife of the federal judge who accepted a package through what she assumed to be the U.S. mail shortly before Valentine's Day. The package contained half a pound of Godiva chocolates. Believing that her husband had sent the candies, she opened the package, ate several of the chocolates, and immediately felt ill. She was transported to a local hospital where she was diagnosed as having tachycardia, not a particularly surprising diagnosis considering that atropine was later identified in her urine. She was subsequently treated and released. The candies, along with the box and packaging material, were recovered and sent to the Federal Bureau of Investigation (FBI) Laboratory for analysis. Upon receipt at the Laboratory, the candies were analyzed for common poisons (cyanide, heavy metals, pesticides, and other chemicals, including drugs). The analyses

determined that the candies contained atropine and pilocarpine, a cholinomimetic drug which is used in the treatment of glaucoma. The detection of these unusual substances confirmed the suspicions of the victim's attending physician. Armed with this information, the investigators searched through the cases over which the judge had presided and developed a list of potential suspects. At about the same time similar packages addressed to a prosecutor's home, as well as at the home of a law enforcement officer, were intercepted. These boxes of Godiva chocolates were examined and found to contain the same drugs. After comparing the three lists of potential suspects, the investigating officers in the case were able to find a common denominator, who was later confirmed via fingerprint analysis of the boxes of candy.

SPOUSAL CONFLICT

Husbands and wives have been ending their relationships by murder since the introduction of the institution. The cases presented below illustrate the extremes to which some individuals will go to disguise a murder, as well as the extent to which life insurance double indemnity can serve as an additional incentive.

In the first case, a middle-aged woman decided that she wanted to end her relationship with her husband, so she poisoned him by placing cyanide in his Extra Strength Excedrin capsules. The husband took the tainted capsules, dying from what was thought to be a natural death from emphysema, and was buried without an autopsy. Had it not been for the wife's greed, the actual cause of death would not have been discovered. However, in order to collect double indemnity on a recently purchased life insurance policy, as well as to be in a position to sue the makers of Excedrin, the victim's wife decided to place other tainted bottles of Excedrin in the market place, so as to be able later to make the claim that her husband had died from the ingestion of a similarly tainted capsule. It is assumed that she perhaps did not intend for more people to die, since she did very little to mask the fact that the bottles had been tampered with (see Figure 1). Unfortunately, the general public is not particularly observant, and as a result another victim took the cyanide-laced capsules and died.

Examination of the bottles removed from retail shelves, along with the bottle recovered from the wife of the first victim, revealed, in addition to the cyanide, green particles that were later identified as ALGAE



Figure 1.

DESTROYER, a commercially available mixture of monuron, simazine, diclone, and atrazine, marketed as an aquarium algicide. Aided by this information, investigators were able to show that the wife of the first victim had purchased the aforementioned product locally, intending it for use in an aquarium. Also local libraries were canvassed for books in the genre of *The Poor Man's James Bond*, with those identified being fingerprinted. In one of the books, on the page dealing with cyanide, her fingerprints were identified.

Approximately 2 months after the Excedrin case appeared in *Readers' Digest*, a similar case, this time however involving Sudafed capsules, surfaced in the same geographical area. As in the Excedrin case, virtually no attempt was made to disguise the tainted product (see Figure 2). This case differed however, in that the intended victim, the wife of a 30-year-old man, did not die; although she did become seriously ill. At least one aspect of the motive was similar in that the husband of



F B I
LABORATORY

Figure 2.

this young woman had purchased a large life insurance policy on his victim shortly before she ingested the dose of cyanide.

PROFESSIONAL CONFLICTS

Professional conflicts, like the incentives explored above, are not novel motives for murder. For example, it was such a situation in the late 1970s, involving 2 physicians at a New Jersey hospital, that led one to employ a quaternary amine to murder the other. These events came to light during a celebrated investigation that became known as the "Dr. X" case.

The first presentation in this discussion involves a conflict between two Ph.D. scientists, and is now known popularly as "The Afrin Affair." In this case a contract between a Texas University and one of the scientists was in the process of termination because it was asserted that he had not published sufficiently in professional literature, and was therefore being classified as nonproductive. Of interest is that the experimenter to be terminated held a number of patents on processing equipment developed for electron microscopy, which were slated to be assigned to the victim scientist upon the subject's departure.

Because the scientist caught up in the termination process felt he had been abused by his colleague, he decided to kill him. His preparation began with the purchase, through a number of legitimate suppliers, of items to include: tetrodotoxin, organophosphates, juniper beans, and botutillium toxin. The plan was to make the death appear to be the work of a terrorist. The ongoing "Desert Shield" and "Desert Storm" operations were to serve as a backdrop. Unfortunately, the campaign in the Middle East was completed before the subject was able to complete his chemical arsenal. He moved ahead with his plan however, selecting beta-propiolactone as his agent, arranging for the compound to be deposited in his victim's bottle of Afrin nasal spray. The victim was able to surmise that something was amiss because of the severe pain associated with the Afrin application. Subsequent examination of the product, as well as the victim's office phone and door handle, revealed the presence of the carcinogen. It is noteworthy that this ostensibly well-read individual, rather than selecting a classical pharmacological text, for example Goodman and Gillman's *The Pharmacological Basis of Therapeutics*, instead sought out the likes of *Uncle Fester's Silent Death*, a reference well-known in underground circles. This cult source is actually quite informative;

the author discusses the dosages of poisons and the pitfalls of using a toxin such as nicotine, which would be easily identified in a routine forensic toxicology screen, as well as extolling the virtues of less well-known substances like beta-propiolactone.

Another illustrative scenario involving a professional conflict is the "Dr. X" investigation. This case involved the use of a skeletal muscle relaxant by Philippine nurses in a Veterans' Administration hospital in Michigan. Although the motive(s) were not completely understood, it is believed that the nurses involved were killing patients either to make other nurses look bad or to euthanize terminally ill individuals. As is many times the case in this kind of investigation, the victims had to be exhumed and the toxins isolated from embalmed tissue, a situation which generally requires specialized extraction schemes as well as detection parameters. This case required the development of a procedure to isolate and identify pancurium from an embalmed environment.

ACCIDENTAL POISONINGS

Both of the cases presented in this discussion involve deaths which occurred during the commission of a felony.

In the first case a Colombian national living in South Florida, died after ingesting the contents of a bottle of "Pony Malta," a nonalcoholic malted beverage imported from Colombia and popular in the Latin communities of Miami, Newark, and New York. The case was originally thought to be an instance of product tampering because the product had been altered in an intentional way after leaving the factory—the bottle contained 50 grams of solubilized cocaine hydrochloride. Following this revelation, some 40,000 bottles of Pony Malta were recalled from retail establishments and screened using a microwave dielectrometer (see Figure 3), so as not to disturb the seals. During this screening event a pattern was discovered—only one bottle in each of the six packs contained cocaine, and the position of that bottle within each six-pack was always the same. Through field investigation, it was determined that the cocaine laden bottles were part of a cocaine smuggling operation. The bottles that made it into retail establishments arrived there because another individual, not associated with the smugglers, broke into the warehouse where the smugglers were recovering the cocaine-tainted bottles and stole bottles of the product



Figure 3.

indiscriminantly. The importers (smugglers) in this case were charged with murder because the aforementioned death had occurred during the commission of the felonious importation of cocaine. Obviously the drug was being imported in this manner in order to circumvent detection. The cocaine was to be extracted from the soft drink via a procedure similar to that employed initially to recover the drug from the coca leaf in the South American jungle. An interesting aspect of the Laboratory investigation of this matter is that the documentation of the poisoning by cocaine was accomplished using hair analysis. The victim, in a coma on arrival at the hospital, was sustained on life support for some 24 days. The initial series of antemortem blood samples (maintained by the hospital) was incomplete at the time of the forensic analysis, and of course the samples taken at the postmortem examination were useless. Head hair was removed from the victim at the time of autopsy, and after an analysis of sequential 5 mm sections, a correlation between the cocaine

concentration as a function of time, and the time of the acute ingestion was made.

A somewhat related case is the "body packing" scheme whereby smuggling is accomplished by the swallowing of condoms or plastic pellets, filled with cocaine or heroin (see Figure 4). The same principle applies: the "bosses" associated with the importation can be prosecuted under U.S. federal law should one of these condoms break and the body packer receive an overdose—the accidental death would be a result of the felonious importation of cocaine.

TERRORISM

Terrorism is the most recently introduced motive for murder. The following two cases deal with the potential use of chemical warfare agents as terrorist weapons.

The first case, although not a state-sponsored

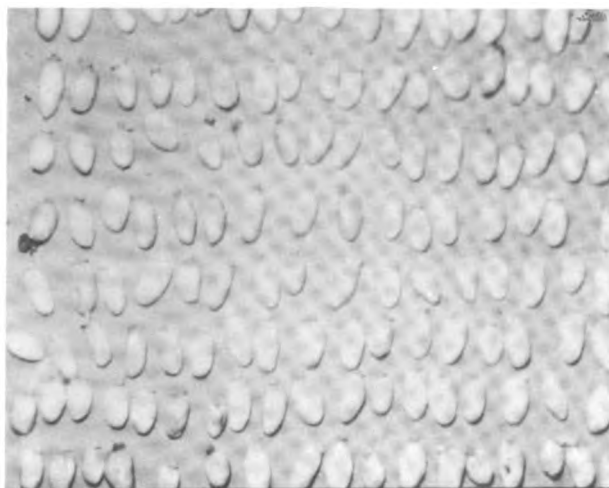


Figure 4.

threat, is still very credible given the subject's knowledge of chemistry. The scenario opens with the receipt of a threat in the form of a letter. In the letter the author refers to phosgene and cyanodimethylaminoethoxyphosphine oxide (Tabun), two well-known chemical warfare agents. The subject failed to follow through on the chemical warfare threats detailed in his letter, instead choosing to kill a federal appeals court judge by sending him a booby-trapped package containing a nail/pipe bomb. The subject was quite capable of synthesizing the items mentioned in his letter, as was demonstrated by the recovery during a search of his home, which netted notes related to synthesis of illicit drugs to include cocaine, methamphetamine, and tetrahydrocannabinol.

The composition of the bomb that he constructed employed a relatively unique explosive—center fire ammunition primer material. It is believed that he chose to use a bomb because of his earlier success with a pipe bomb attack on his first wife. The link between the author of the letter and the subject was made via some personal effects found during a search of his home. Specifically, Tabun's complete chemical name is unusual in addition to being somewhat unwieldy. This particular term is encountered in virtually only one source, that being a particular edition of Van Nostrand's Scientific Encyclopedia, a copy of which was found at the subject's

home. Interestingly, the name was misspelled the same way in both the textbook and the letter.

Another related investigation is the attempt to assassinate then President of the United States, George Bush, during the Gulf War. In this case a Ph.D. chemist, who was employed by a chemical company in the southern area of the United States, contacted the Iraqi Embassy in Washington, DC, by telephone and offered to synthesize nerve agents for them. In the telephone conversation, the subject suggested that these agents be deployed against the President during his State of the Union address, which was to take place during the Gulf War hostilities. This individual, like the previous gentlemen, was quite capable of carrying out the synthesis—the laboratory which employed him was involved in the manufacture of organophosphate pesticides, compounds very similar to nerve agents. This particular individual was foiled in his assassination attempt primarily due to creative investigative techniques.

SUMMARY

The discussions presented illustrate the need to continue full toxicological screens in all investigations of the potential ingestion of a suspicious substance, so that the successful application of an unusual poison does not go unreported. In addition, it should be noted that because of the generally inadequate specimens usually received by forensic laboratories, both in type as well as volume, more efficient interaction between investigators and toxicologists is crucial if cases similar to the ones discussed herein are to be solved.

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Atmospheric Pressure Ionization Combined with LC/MS: Emerging Technologies in Forensic Toxicology

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Mass spectrometry (MS) may be the ultimate detection when combined with modern condensed phase separation sciences. The technique combines sensitivity with excellent specificity, so the forensic analyst can obtain definitive information regarding components separated in a mixture. Thus, mass spectrometric detection not only provides evidence of a peak, but it also provides important clues including molecular weight and structural information enabling identification of the components. The coupling of an atmospheric pressure ionization (API) mass spectrometer to most of the separation science techniques offers a simpler alternative from earlier nonroutine, less sensitive systems where the vacuum systems struggled to handle the liquid effluent from these systems. Today's sensitive and analytically rugged API systems can be operated unattended for extended periods of time, thus reducing the cost per sample to a reasonable value especially given the wealth of information provided. Although the mass spectrometer is more complicated than conventional spectroscopic detectors, today's API systems effectively decouple the liquid phase separation inlet from the high-vacuum system where mass analysis occurs. The ability to form gas-phase ions at atmospheric pressure and sample primarily the analyte ions into the mass spectrometer promises a bright future for combined on-line separation mass spectrometry with modern separation science techniques. The ease of performing these experiments offers new analytical opportunities for forensic laboratories.

INTRODUCTION

The success and general acceptance of capillary gas chromatography/mass spectrometry (GC/MS) in forensic urine drug testing attests to the importance and need for on-line separation mass spectrometric detection of organic compounds. Unfortunately, many compounds are not amenable to gas chromatography (GC) conditions even with modern derivatization techniques and column technology due to their thermal instability, highly polar nature, or high molecular weight. When this is the case, many practicing analysts resort to high performance liquid chromatography (HPLC) techniques using ultraviolet (UV), fluorescence, or other detectors. More recently, ion pairing techniques (Gupta and Dasgupta 1988) and capillary electrophoresis (CE) (Gordon *et al.* 1988) have been used to separate mixtures containing a wide variety of organic compounds. Many of these mixtures include compounds that are not amenable to GC analysis because they contain peptides, proteins, or charged species.

HPLC techniques are now ubiquitous in academic and industrial laboratories and CE is receiving considerable interest as instrumentation becomes commercially available. Unfortunately, however, the practicing HPLC or CE analyst soon returns to the frustration and uncertainty experienced in the GC/FID days before GC/MS was available where a peak and its retention time are the only information available. At this stage one must either coinject a sample of the suspect

compound after guessing what it is, or preparatively collect the peak and perform further characterization. Clearly, it is just as advantageous to have a mass spectrometer detector for HPLC and CE as for GC.

Although a variety of combined liquid chromatography/mass spectrometry (LC/MS) systems have been reported (Covey *et al.* 1986; Yergey *et al.* 1990), we suggest that API mass spectrometry is a preferred way to simplify the coupling of liquid inlet systems such as HPLC, CE, and ion pair chromatography to mass spectrometry (Huang *et al.* 1990). A key feature of this approach is that liquid effluent from a particular separation science is not directly introduced into the mass spectrometer vacuum system. As a result, the mass spectrometer high vacuum pumps do not have to deal with removing the solvent and buffer additives while enriching the analytes of interest. As shown in Figure 1, the effluent is sprayed in the vicinity of an ion sampling orifice that effectively separates high vacuum from atmospheric pressure. Gas-phase ions are formed in this region by either electrospray (Fenn *et al.* 1989) or atmospheric pressure chemical ionization (Huang *et al.* 1990), and then these ions are sampled through the ion sampling orifice into the vacuum system for mass analysis. Large excesses of solvent from the effluent do not enter the vacuum system, thus making life much easier for the mass spectrometer hardware and pumping system.

Many different classes of compounds may be detected via this approach provided the analyst has a

few different "tools" in his or her toolbox. For example, the mass spectrometer should be capable of detecting both positive and negative ions. Thus, when the proton affinity of an analyte is sufficient to allow protonation, a positively charged ion is produced requiring positive ion detection. When negatively charged ions are the more stable species such as sulfonate, carboxylate, or phenolate ions, then negative ion detection is preferred. Sometimes either mode of detection may be possible, but usually one will provide much better sensitivity.

In addition to being able to detect either cations or anions it is useful to have more than one interface for coupling a separation technique to API mass spectrometry. An interface is typically a simple, relatively inexpensive probe-type device that may be placed in front of the ion sampling orifice of the mass spectrometer. It usually is designed to provide a particular type of gas-phase ionization and/or handle different effluent flow from the condensed phase separation system. For example, a pure electrospray interface coupled to an API mass spectrometer is optimized with eluent flows in the 1 - 5 $\mu\text{L}/\text{min}$ range. This confines one to using either open tubular HPLC, packed capillaries, or a significant postcolumn split from larger bore HPLC systems. In contrast, the pneumatically-assisted electrospray or ion spray LC/MS interface (Bruins *et al.* 1987) increases the usable flow rate range of electrospray to 40 - 50 $\mu\text{L}/\text{min}$ or, with some reduction in sensitivity, to 100 - 200 $\mu\text{L}/\text{min}$. This allows routine LC/MS operation using 1 - 2 mm i.d. microbore columns with total effluent introduction

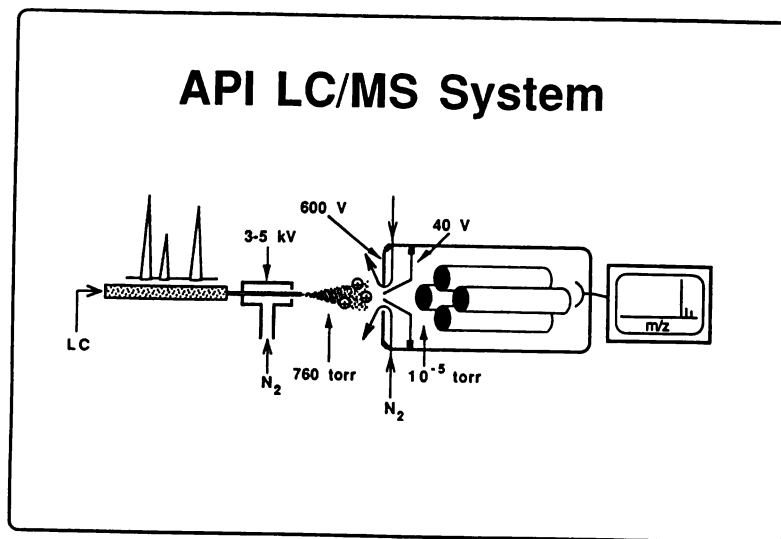


Figure 1. Schematic of a generic liquid inlet APIMS system. (Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.)

or conventional HPLC columns with some postcolumn split of the total effluent.

In contrast to problems dealing with too much effluent from HPLC columns the technique of CE with electroosmotic or bulk flow ranging from zero to a few nanoliters per minute poses different challenges. In this instance a makeup flow of buffer must be provided sufficient to sustain a stable spray from either electrospray or ion spray. Smith *et al.* (1988) have described a sheath-flow device that delivers a makeup flow coaxially at the CE column terminus. In contrast, a liquid junction device has been described that is placed between the CE column exit and the ion spray interface (Lee *et al.* 1989). Both devices provide the opportunity for decoupling to some extent the running buffer conditions necessary for the CE process from the preferred and oftentimes different buffer conditions for optimum performance from the electrospray ionization process.

The flexibility afforded by the API mass spectrometric approach stems in part from its ability to accommodate a variety of different LC/MS-type interfaces to one mass spectrometer. One can couple several different separation science techniques to the same mass spectrometer simply by utilizing the appropriate interface. This requires no modification of the chromatographic or CE hardware or the mass spectrometer. It may, however, necessitate some compromise of the chromatographic eluent or CE buffer which basically requires the avoidance of inorganic modifiers and buffer concentrations greater than 20 mM. The analyst should appreciate that any ionized species in the effluent will likely be detected by the mass spectrometer. Depending upon their molecular weights these components may contribute high chemical noise or interfere with detection of either target or unknown analytes.

Aspects and examples of combining condensed phase separation techniques with API mass spectrometric detection will be described in this paper. The strengths and limitations of the techniques will be highlighted from the viewpoint of both the separation science and mass spectrometry. We will address the current status of API LC/MS and related techniques, describe where the technology is going in the future, and discuss the potential limitations of the techniques from the perspective of the separation scientist and the mass spectroscopist.

EXPERIMENT

Mass Spectrometry

This work describes the use of two different quadrupole mass spectrometer systems to obtain the results described. Mass spectrometer I is a TAGA 6000E upgraded to an API III tandem triple quadrupole (Sciex, Thornhill, Ontario), and mass spectrometer II is a modified single quadrupole 5970 Mass Selective Detector (Hewlett-Packard Co., Palo Alto, CA). The first system is a standard commercially available API instrument with a nitrogen gas curtain feature and a mass range to 2,400 Da. The second instrument does not require a curtain or bath gas (Duffin *et al.* 1992) feature and has a mass range to 800 Da. However, it is a significantly modified benchtop GC/MS system that accommodates sampling gas-phase ions formed at atmospheric pressure, and is not yet commercially available. The ion spray and related interfaces may be used with either of these systems. System I can provide true collision-induced dissociation (CID) (Busch *et al.* 1988) in the central quadrupole collision cell, while the single quadrupole System II provides CID in the free-jet expansion region in the first pumping region prior to mass analysis. During CID experiments mass spectrometer I maintains an analyzer vacuum of 2.5×10^{-5} Torr. Argon is used for CID in the collision region (second quadrupole) with 2×10^{14} atoms/cm² target gas thickness. The precursor ions are formed following their field-induced desorption from the condensed phase, transmitted to the second quadrupole for CID, and the resulting product ions mass-analyzed in the third quadrupole to provide a CID mass spectrum. Both mass spectrometer systems are operated with unit mass resolution in either the full-scan acquisition or selected ion monitoring (SIM) modes with either positive or negative ion detection.

The condensed phase separations described on-line with mass spectrometry in this report include HPLC and CE. An example of each will be described in sequence using the commercial API mass spectrometer I system followed by an example of LC/MS performed on the modified MSD benchtop mass spectrometer system II. It is the view of the authors that this last example offers considerable potential to the practicing separation scientist in the future because of its simplicity, potential low cost, and analytical flexibility. However, the tremendous analytical potential of the more

sophisticated tandem mass spectrometry (MS/MS) system will continue to be of considerable value for more challenging problems.

MICRO LC/MS USING A COMMERCIAL API MASS SPECTROMETER

Apparatus

The HPLC system for LC/MS included a 1 mm i.d. x 100 mm C-18 bonded, 5 μ m particle-size packed column coupled to a ABI Model 140 dual-syringe micropump (Applied Biosystems Inc., Santa Clara, CA) equipped with a Model 9125 biocompatible syringe-loading sample injector (Rheodyne, Cotati, CA). The micro HPLC pumping system maintained an eluent flow of 40 μ L/min while the gradient began at 0% B and was programmed directly to 10% B over the first minute followed by a slow gradient program to 90% B over 24 min following the injection of 20 μ L sample (A = 0.05% TFA in water; B = 0.05% TFA in 50/50 acetonitrile/water). The column exit was connected directly to a Model 757 micro UV detector set at 215 nm (Applied Biosystems, Inc.) followed by the ion spray interface that accepted the total micro HPLC effluent. The Sciex API III mass spectrometer was operated in the full-scan, single MS mode with positive ion detection.

The sample of sulfonated RNase B was prepared according to the procedure of Thannhauser *et al.* (1984). The crude sample was desalted with 50 mM ammonium bicarbonate with a PD-10 (Pharmacia, Uppsala, Sweden) column, and digested with TPCK-treated trypsin (Sigma Chemical Co., St. Louis, MO) using a 50:1 substrate:enzyme ratio at 37 degrees for 12 h. Aliquots of this sample (20 μ L) were injected on-column for LC/UV/MS analysis.

Results and Discussion

Micro LC/MS techniques are useful for the sensitivity and complex mixtures containing low levels of analytes of interest. Since there is some concern that peptides and related compounds may be among the drugs of the future, we have explored new methods for the characterization of these polar, higher molecular weight compounds. It is generally accepted that capillary GC/MS techniques are not well suited for the analysis of samples containing peptides and glycopeptides, so we have evaluated the analytical potential of micro LC/

S techniques for this purpose. As an example we are interested in the characterization of the carbohydrate composition of glycoproteins such as RNase B, and have studied the products resulting from the sulfonation of the disulfide bonds followed by tryptic digestion. These tryptic peptides are representative of larger biomolecules that may be important in the forensic arena in the future. The LC/UV chromatogram (215 nm) from the analysis of the tryptic digest of S-SO₃-RNase B is shown in Figure 2A. This shows the chromatographic separation of a complex mixture over a period of about 25 min under the reversed-phase micro HPLC conditions described. The injection of approximately 1 nMol of the digest material provides evidence for even weakly-absorbing components in the mixture. LC/UV identification of any peaks of interest would require laborious experiments designed to compare retention times between a known compound and a peak of interest in Figure 2A. The

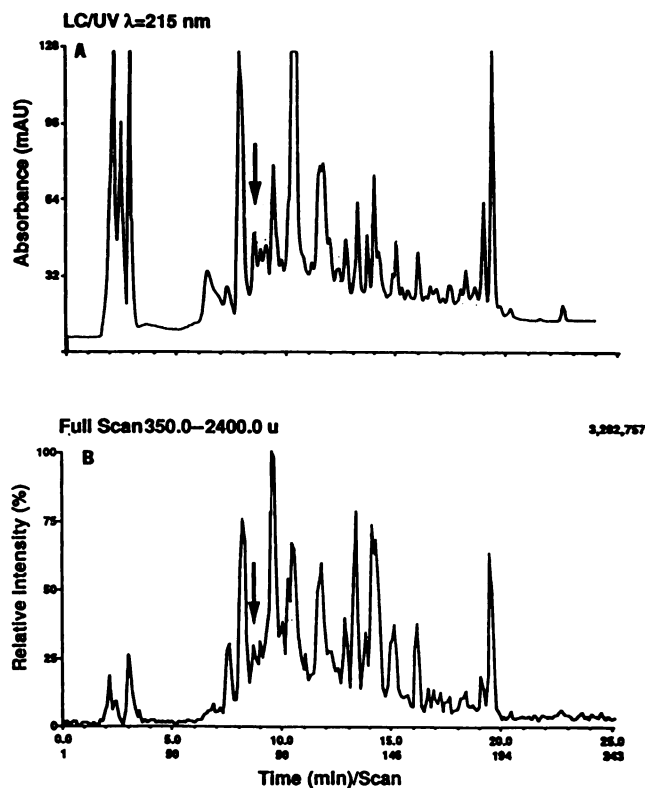


Figure 2. A) LC/UV; and B) LC/MS TIC from the analysis of 1 nMol from tryptic digest of S-SO₃-RNase B. Full-scan LC/MS data were acquired from m/z 300 - 2400 in 1 Da steps with a 2.86 msec dwell time per step. The TIC is plotted from 350 - 2400 Da to give an improved S/N ratio. See text for HPLC and gradient conditions. (Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.)

correct choice of the "known compound" can be very problematic since complex mixtures such as this can contain many surprises.

An alternative to the trial and error approach mentioned above is the acquisition of on-line LC/MS results while scanning the mass spectrometer across its available mass range so the molecular weights of all components detected may be determined. The total ion current profile shown in Figure 2B was obtained for this sample by monitoring the total micro HPLC effluent while scanning the first quadrupole from m/z 300 - 2,400. Each chromatographic peak represents one or more tryptic peptides or glycopeptides. Inspection of the full-scan mass spectrum for any of these peaks provides the molecular weight for each component in the form of its doubly-protonated molecule ion. The mass spectrometer may be tuned to provide little or no fragmentation so that only the molecular weight species are observed, or sufficient energy may be imparted to the ions as they pass through the ion sampling orifice that some fragmentation occurs. In the latter case simple structural information may be available in addition to the molecular weight information. In most all cases, however, the molecular weight may be determined from these experiments. This information is very useful for the characterization of the composition of a complex mixture such as that shown in Figure 2A.

Figure 3 shows a representative mass spectrum obtained from the indicated 8.7 min retention time region shown in Figures 2A and 2B. These LC/MS data reveal the coelution of several doubly and triply-protonated glycopeptides consisting of differing numbers of mannose linkages eluting in the same retention time window. For example, if X represents the number of mannose linkages on the doubly-protonated, doubly-charged glycopeptide shown in Figure 3, these data suggest the presence of compounds that differ by 5 - 9 mannose linkages on the NLTK-chitobiose core glycopeptide. Similarly, if Y represents the number of mannose linkages on the triply-protonated, triply-charged glycopeptide, we see that the number of glycopeptides with different numbers of mannose linkages ranges from 7 to 9. The absence of ions where $Y = 5$ and 6 in this ion series presumably is due to their weak abundance in the triply-protonated, triply-charged ion series.

Although these LC/MS data do not reveal directly the identity of all the components observed, they do provide considerably more information than can be obtained from the LC/UV chromatogram shown in Figure 2A. For example, several minor components are observed in Figure 2B that would not have been suspected from LC/UV results. Additional protocol utilizing tandem mass spectrometry (LC/MS/MS) can

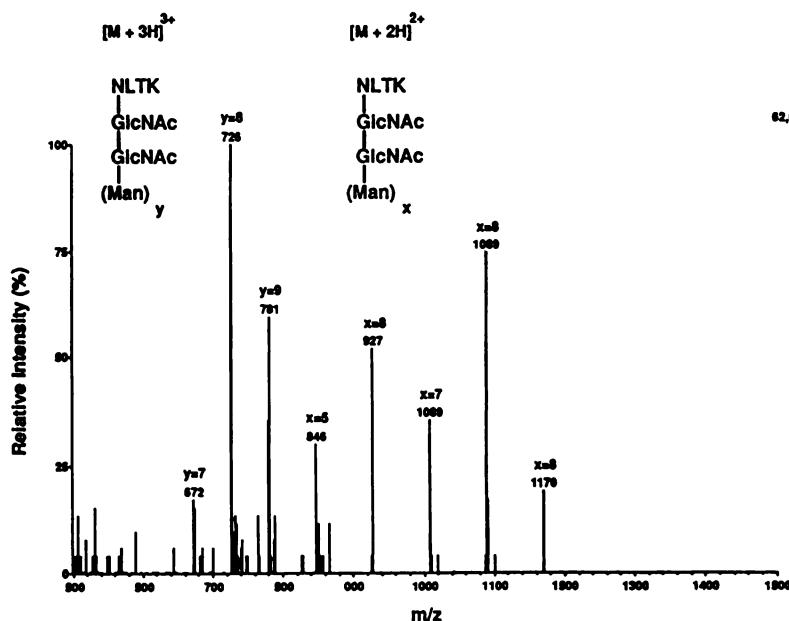


Figure 3. Full-scan LC/MS spectrum of glycopeptides with varying mannose compositions observed at a retention time of 8.7 min in Figure 2B. The doubly-protonated, doubly-charged (x -series) ions and the triply-protonated, triply-charged (y -series) ions show the distribution of coeluting glycopeptides present in the digest mixture. See text for more detailed discussion. (Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publication, A Division of Preston Industries, Inc.)

be utilized to obtain more structural information such as the product ion fragmentation behavior of the multiply charged precursor ions. Since CID of doubly-charged precursor ions of tryptic peptides give primarily singly charged product ions, the interpretation of these data can be quite informative. These and related LC/MS/MS experiments may be easily utilized when one has a reliable LC/MS interface and a tandem mass spectrometer.

CAPILLARY ELECTROPHORESIS/MASS SPECTROMETRY (CE/MS)

Apparatus

A homemade CE system was used in this work. The column was untreated fused-silica (75 μm i.d. x 35 cm) that was connected directly to a liquid junction interface. The latter was directly connected to an ion spray interface that was operated as described above. These combined units are shown in Figure 4 and are described in more detail elsewhere (Lee *et al.* 1989). The liquid junction along with the ion spray interface floats at 3 kV. The potential difference across the CE column is the difference between the applied potential at the capillary inlet end and the voltage applied to the liquid junction-ion spray interface combination. The key features of the liquid junction coupling device include a means of decoupling the separation requirements of the running buffer from the buffer

requirements necessary for *optimal* ion spray operation. In addition, the liquid junction provides a *makeup* flow for the very low electroosmotic or bulk flow of the running buffer. This device is used instead of the sheath flow approach described by others. The running buffer used in this work was 5 mM ammonium acetate in 25% acetonitrile adjusted to pH 5 with acetic acid. The makeup buffer in the liquid junction reservoir was 25% acetonitrile in 5% formic acid, and an applied voltage of 38 kV was placed at the inlet (anode) end using a Model 230-30R series reversible polarity high voltage power supply (Bertan Assoc., Inc., Hicksville, NY) while the exit (cathode) end was maintained at the ion spray interface voltage of +3 kV. This produced a potential difference across the capillary column of 35 kV. Samples were loaded into the inlet of the capillary via hydrostatic injection by elevating the inlet 6 cm for 5 s. The 6 cm distance was measured between the liquid surface of the sample vial and the surface of the liquid junction buffer reservoir. The API mass spectrometer used was the same as described above, and was operated in the SIM mode for these experiments.

Results and Discussion

Although a variety of CE applications have appeared in the recent past using UV (Terabe *et al.* 1984), fluorescence (Jorgenson and Lukacs 1981), and indirect fluorescence (Kuhr and Yeung 1988), there have been relatively few reports of on-line CE/MS. The two

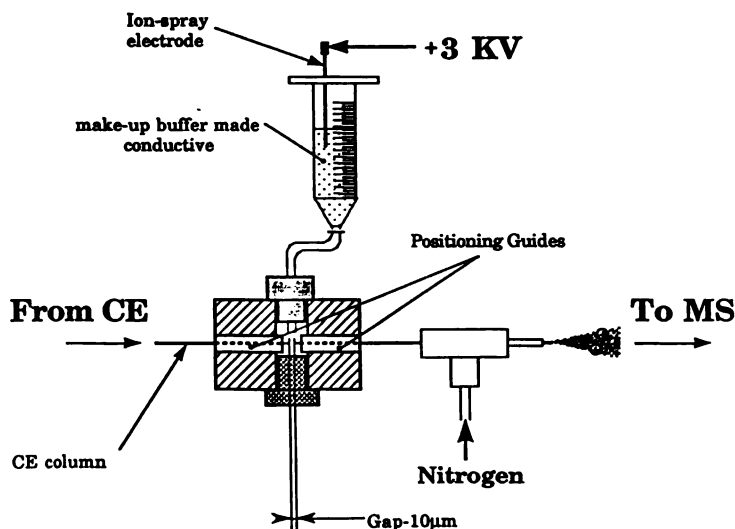


Figure 4. Combined liquid junction-ion spray interface for CE/MS applications. The potential across the capillary column is the difference between that applied at the inlet end and the potential applied in the makeup reservoir of the liquid junction. The ion spray interface floats at the same potential as the liquid junction by conduction through the buffer electrolyte. (Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.)

approaches to CE/MS to date include those performed using continuous flow fast atom bombardment (CFAB) (Caprioli 1990) and electrospray (Fenn *et al.* 1989). CE/MS via CFAB has been reported using two different approaches (Caprioli *et al.* 1989; Mosely *et al.* 1991), and CE/MS via electrospray has been reported using the sheath-flow (Smith *et al.* 1988) and the liquid junction devices (Lee *et al.* 1989). Neither approach to CE/MS is trivial to accomplish, but there is a developing opinion that the latter approach is more practical experimentally. Our recent experience with a commercial P/ACE 2050 CE system (Beckman Instruments, Inc., Palo Alto, CA) and a homemade system using the above-described liquid junction-ion spray interface suggests that CE/MS techniques may play a significant role in the future of analytical chemistry. The recent commercial availability of CE instruments that provide an automated, instrumental format for electrophoresis promises to complement the long, favorable history of slab gel electrophoresis.

An example of on-line CE/MS using the combined liquid junction-ion spray apparatus is shown in Figure 5. In this example 8 pmol per component of a synthetic mixture containing sulfonylurea herbicides were loaded onto the inlet end of the capillary. The mass spectrometer was operated in the SIM mode with positive ion detection. Thus, the protonated molecule ions for each component were monitored at m/z 365,

396, 411, and 415. The molecular weights of these compounds in their order of elution in Figure 5 are 410, 364, 395, 410, and 414. These data reveal the quality of separation efficiency provided, and relatively high speed of separation from the CE/MS technique described here. In contrast to our earlier report (Huang *et al.* 1990) where it took over 24 min to separate 5 sulfonylurea compounds, the shorter CE column used for the data shown in Figure 5 demonstrate the separation in less than 4 min. Additional structural information is available using tandem mass spectrometry techniques such as CID of the precursor ions while scanning the third quadrupole through the full mass range to observe all the product ions formed. This capability is a useful adjunct to the ion spray CE/MS technique which by itself only provides molecular weight information.

Because the CE liquid inlet system is physically separate from the APIMS vacuum system, coupling these two analytical techniques is straightforward. In contrast, the CFAB approach to CE/MS requires that the exit of the CE capillary or a connecting capillary from 0.5 - 1.0 m long be directed inside the vacuum system of the mass spectrometer. The vacuum system thus pulls on the exit of the capillary and can contribute to band broadening and other adverse effects unless the latter is decoupled from the separation capillary (Caprioli *et al.* 1989; Mosely *et al.* 1991). In addition, the CFAB

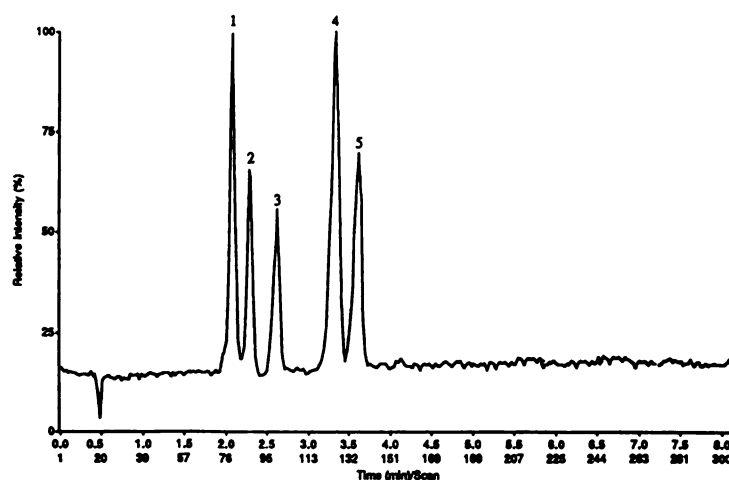


Figure 5. SIM CE/MS total selected ion current electropherogram for 2 pmol per component of a synthetic mixture containing sulfonylurea herbicides. The separation conditions were: 75/25 $\text{CH}_3\text{CN}/5\text{mM NH}_4\text{OAc}$, pH = 5. The sample was loaded into the capillary column hydrostatically by elevating the inlet 6 cm for 5 s while the column inlet was immersed in the sample. The capillary was uncoated fused-silica, 75 μm i.d. x 35 cm. The anode was held at 38kV while the liquid junction was held at 3 kV to give a potential across the column of 35 kV. The peak elution order is: 1) bensulfuron methyl, MW = 410; 2) sulfomethuron methyl, MW = 364; 3) tribenuron methyl, MW = 395; 4) nicosulfuron, MW = 410; and 5) chlorimuron ethyl, MW = 414. (Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.)

approach requires a matrix of glycerol or related material to facilitate the FAB ionization process. This material must be continuously introduced into the mass spectrometer either coaxially (Mosely *et al.* 1991), or via a makeup junction along with the running buffer to provide continuous ionization (Caprioli *et al.* 1989). Sometimes additional material must be added to the matrix to provide uniform response for all analytes of interest (Caldwell and Gross 1989). These requirements for CFAB combined with CE place stringent practical demands for accomplishing routine CE/MS. Although impressive CFAB CE/MS results have been reported, it appears that the electrospray ionization approach to CE/MS may be more widely used in the future.

A limitation remains, however, for CE/MS and CE/MS/MS due to the low sample capacity of the CE capillary column, and the limited sensitivity afforded by today's mass spectrometers. In addition, sometimes it is necessary to compromise on the buffer used to achieve adequate sensitivity from the electrospray ionization process. Although SIM techniques provide better sensitivity than full-scan CE/MS experiments, our best performance has struggled to detect low fMol quantities of well-behaved small peptides (Lee *et al.* 1989), and typically we are limited to high fMol to low pMol CE/MS detection capability. In contrast, others have demonstrated much better detection limits using, for example, indirect fluorescence detection (Kuhr and Yeung 1988). Even UV detection typically has better

detection limits for well-behaved compounds than present MS capability. This situation may change, however, as ion trap mass spectrometers are adapted to CE/MS capability (*vide infra*).

ION SPRAY LC/MS ON A BENCHTOP MASS SPECTROMETER

Apparatus

The micro HPLC system used for LC/MS was a Model 600 MS (Millipore) operated under isocratic conditions at 40 $\mu\text{L}/\text{min}$. The eluent was 5 mM ammonium formate in 70/30 acetonitrile/water. The column used for the elution of the indole alkaloid, reserpine, was a 1 mm i.d. x 100 mm Zorbax RX-C-8, 5 μm particle-size custom-packed by Keystone Scientific (Bellefonte, PA). A fixed-loop (1 μL) Rheodyne Model 7520 syringe-loading sample injector was used to inject the sample onto the microbore HPLC column. The total effluent was directed through the ion spray interface and the spray region of the interface was positioned near the ion sampling capillary of the modified MSD mass spectrometer (Figure 6).

The modification and performance of the Hewlett-Packard 5970 MSD single quadrupole mass spectrometer has been described elsewhere (Duffin *et al.* 1992). Basically, the GC and the entire standard electron ionization (EI) ion source-lens system were

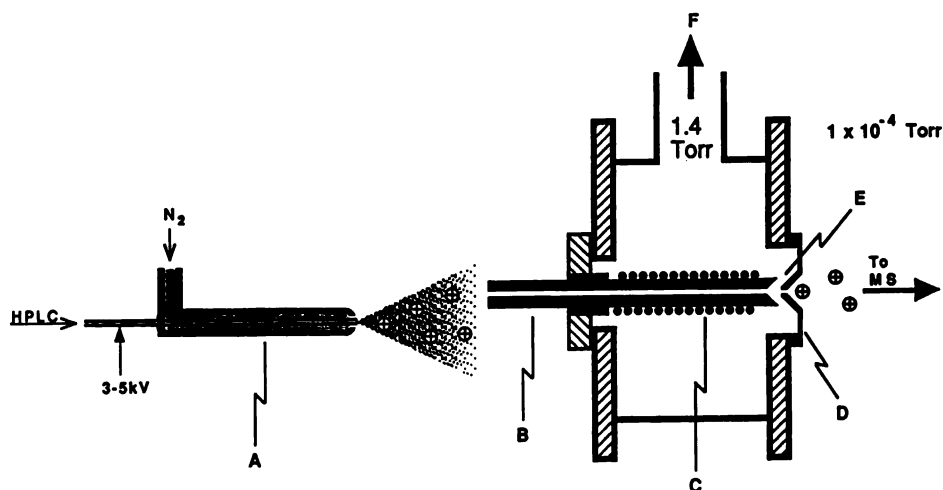


Figure 6. Ion spray API MSD combination inlet system on a modified Hewlett-Packard 5970 mass selective detector. A) Ion spray interface; B) ion sampling capillary; C) heated region (80°C) of ion sampling capillary; D) conical ion sampling orifice separating the first vacuum stage from the high vacuum mass analyzer region of the single quadrupole mass spectrometer; E) CID region where ion-molecule interactions can be utilized to produce fragmentation of protonated molecule ions; F) rotary pump outlet to evacuate the first stage of pumping to 1.4 Torr. (Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.)

removed from the MSD. The analyzer housing was replaced with one fitted with a 4 in flange that accommodates a larger (330 L/sec) turbo molecular pump. A two-stage API sampling inlet for the high vacuum system was fabricated with an ion sampling capillary similar to that reported by Chait *et al* (Katta *et al.* 1991). A heated ion sampling capillary (500 μm i.d. x 14 cm) provides differential pumping between atmospheric pressure and an intermediate 1.4 Torr region (Figure 6). Ions formed at atmospheric pressure via the ion spray interface (Figure 6A) are sampled through the ion sampling capillary (Figure 6B) and enriched relative to gas molecules in the region between the capillary tube exit and the ion sampling conical orifice (Figure 6E). The latter (Figure 6D) separates the intermediate 1.4 Torr region from the high vacuum (1×10^{-4} Torr) quadrupole mass analyzer region. By applying appropriate voltage potentials on the ion sampling capillary and the ion sampling conical orifice, ions can be focussed into an Rf-only quadrupole lens that is capacitively coupled to the standard MSD mass analyzer (not shown in Figure 6). Minor adjustment of the standard Rf-scan coils allows the combined Rf-only-mass analyzer quadrupole system to function in tandem.

Results and Discussion

The system described above has been used to analyze samples containing environmentally important pesticides, benzodiazepines, and their metabolites in human urine, LSD, and tetraalkyl ammonium compounds (Duffin *et al.* 1992). Recent results in the negative ion mode of detection have shown feasibility for the detection of inorganic anions such as nitrate, sulfate, and halides with this system. The LC/MS data shown in Figures 7 and 8 were obtained from the analysis of the potent tranquilizer drug, reserpine. This thermally unstable basic drug is not amenable to GC/MS characterization yet very sensitive analytical capability is required to detect its presence in biological fluids such as equine plasma (Akbari *et al.* 1986). In an effort to determine whether the benchtop LC/MS system could be used to regularly monitor reserpine in such biological fluids, we have begun preliminary ion spray LC/MS investigations with this system. Figure 7 shows a full scan CID mass spectrum obtained from the on-line LC/MS analysis of a 600 ng sample of reserpine injected onto the 1 mm i.d. micro HPLC column. This rather

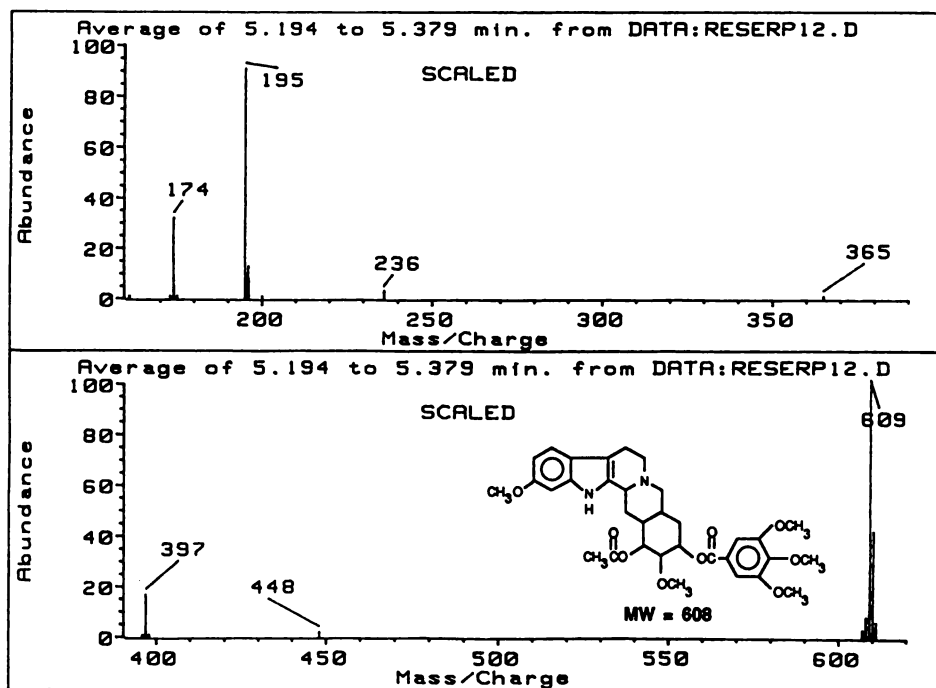


Figure 7. Unabstracted full-scan CID mass spectrum from the on-column LC/MS analysis of a 600 ng sample of reserpine. A potential difference of 100 volts was applied between the ion sampling capillary (Figure 8B) and the conical ion sampling orifice (Figure 8D). (Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.)

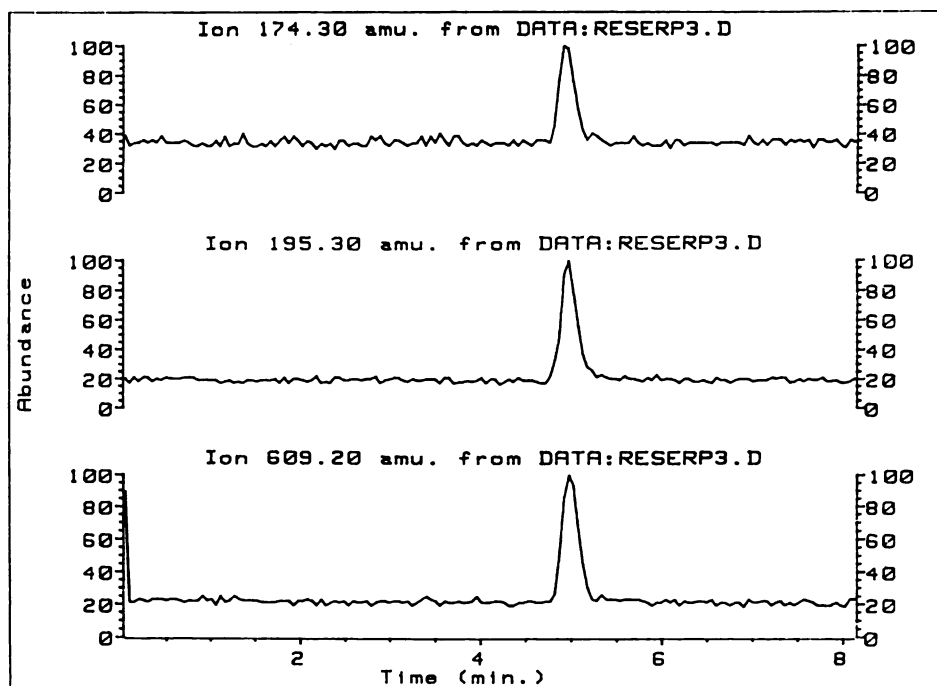


Figure 8. SIM CID LC/MS selected ion current profiles for m/z 174.3, 195.3, and 609.2 $[(M+H)^+]$ following on-column injection of 1.5 ng reserpine. See text for HPLC conditions. (Reproduced from the *Journal of Chromatographic Science* by permission of Preston Publications, A Division of Preston Industries, Inc.)

high level of sample was used initially for a preliminary determination of the optimum tuning parameters for this analyte. The CID mass spectrum shown in Figure 7 is not a classical MS/MS mass spectrum. Instead, the mass spectrum in Figure 7 was obtained by increasing the potential difference between the ion sampling capillary (Figure 6B) and the ion sampling cone (Figure 6D) in the first vacuum region. This increases the energy of ion-molecule collisions in this region thus causing fragmentation of the protonated molecule ion for reserpine at m/z 609. This process differs from the true MS/MS situation where there is mass analysis prior to the CID process. Thus, this "poor man's" CID does not provide mixture analysis as does MS/MS, but it does provide structural information by producing a full-scan mass spectrum characteristic of the compound's structure. Figure 7 is not an EI mass spectrum, but there are similarities between it and a conventional EI mass spectrum from reserpine (Mills *et al.* 1984). For example, the m/z 448, 397, 365, 195, and 174 ions observed in Figure 7 are also present in the EI mass spectrum of reserpine although the latter has additional ions not observed in our CID mass spectrum.

After optimizing the ion spray benchtop CID LC/MS conditions for reserpine, characteristic fragment ions from Figure 7 may be selected and monitored in the

SIM mode in subsequent CID LC/MS experiments. By monitoring only 3 abundant, structurally important ions [e.g., m/z 609 $(M+H)^+$, 195, and 174], the mass spectrometer does not waste time scanning regions in the mass scale where there is no significant information. These SIM experiments can provide significantly improved sensitivity. Figure 8 shows the results from such a SIM CID LC/MS analysis of a standard reserpine sample when 1.5 ng of the compound are injected on-column. The 3 panels in Figure 8 show the ion current profiles (from top to bottom) for m/z 174.3, 195.3, and 609.2. The signal-to-noise ratio for these data is very good suggesting that subnanogram detection limits may be possible for this compound with the current system. We are currently working to improve the sensitivity of this system further and believe that another factor of 10 improvement should be possible. These results will be presented subsequently.

SUMMARY AND CONCLUSIONS

This report has presented results demonstrating that an API mass spectrometer is an effective and useful detector for HPLC and CE. Both conventional HPLC and micro HPLC flow rates have been described as well as submicroliter per minute flow in the case of CE. In addition, two different mass spectrometers and three

different "liquid inlet" systems housed in the same laboratory were used by at least three different people to obtain the results shown. Another related but different LC/MS interface, the heated pneumatic nebulizer device, is also routinely used although results from this interface are not described in this work (Huang *et al.* 1990; Wachs *et al.* 1991). It is usually implemented for applications involving less polar analytes, and may be used with either of the mass spectrometers described (Huang *et al.* 1990; Duffin *et al.* 1992). The routine use of these systems occurs in this laboratory for basic and applied research as well as specialized service. Both qualitative (Muck and Henion 1990) and quantitative (Weidolf *et al.* 1988) results are possible in addition to coupled column HPLC combined with MS/MS (Edlund *et al.* 1989). These techniques have all been demonstrated and may be implemented by the appropriately initiated and qualified personnel.

In many laboratories and areas of analytical chemistry high sensitivity and trace analysis capability remains an elusive goal. The techniques described in this paper do not currently satisfy all requirements of an ultrasensitive technique, but progress is being made towards this goal. For example, ion trap technology originally available for GC/MS applications continues to look very promising. When one considers recent developments with the quadrupole ion trap mass spectrometer (Cooks *et al.* 1991) (Finnigan MAT, San Jose, CA) it becomes apparent that this detector could add significantly improved capability over what we now have from more conventional mass spectrometers. The recent report by Cooks *et al.* has demonstrated high sensitivity, high mass resolution, and multiple stages of MS/MS (Nourse and Cooks 1990). The dramatically increased sensitivity shown here may be a significant benefit towards matching the low sample capacity demands of CE/MS described above. When this capability is combined with electrospray (Van Berkel *et al.* 1990) and becomes routinely available on commercial systems, on-line LC/MS analyses using ion trap technology (McLuckey *et al.* 1991) may be able to provide much better analytical capability than is available today. In addition, the quadrupole ion trap hardware is inherently more simple, and presumably could be significantly less expensive than today's benchtop systems. This could, of course, make it even easier to implement this analytical capability into more laboratories. Progress is being made in our laboratory

as well as in other laboratories to implement electrospray ionization on simple, inexpensive ion trap systems. Surely the future is bright for dramatic improvements in the ease of use and performance of these benchtop technologies. These improvements will make forensic LC/MS techniques more available and thus better prepare laboratories for the challenging analytical demands of the future.

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Forensic Toxicology: Today, Tomorrow, and Beyond

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Forensic toxicology has been defined as the study and practice of the application of toxicology to the purposes of the law and the just resolution of social and legal issues. The broad term toxicology deals with many aspects of the toxicity of various drugs and chemicals. This can include issues dealing with the environment and the development and manufacture of new pharmaceuticals, including the adverse affects of commonly prescribed and over-the-counter drugs. Forensic toxicology is that part of toxicology which includes the detection and measurement of drugs and chemicals in biological fluids and tissues and the evaluation of the expected and untoward effects of these chemicals. Correlation and interpretation of such findings emphasize the fact that the results may be predesignated for use in various judicial, legal, or administrative settings. Toxicology imports many other fields. These include various aspects of chemistry, such as analytical chemistry and organic chemistry, pharmacology, physiology, pathology, and other aspects of medicine. Forensic toxicology is an objective applied science. In the medical examiner situation, it functions as part of a triad which includes investigation, autopsy and medical findings, and laboratory testing.

Forensic toxicology is one of the older of the forensic sciences, along with forensic pathology and early aspects of forensic criminalistics. The forensic sciences encompass both fact and opinion. This is illustrated by the nine functions stated in Figure 1. This example, taken from medical examiner experiences, illustrates the combination and utilization of both fact and opinion expected of the forensic toxicologist.

THE VARIED FACETS OF FORENSIC TOXICOLOGY

Forensic toxicology has generally been divided into three areas: postmortem or medical examiner toxicology, human performance or driving under the influence of alcohol and drugs, and forensic urine drug testing in the workplace. However, forensic toxicology may encompass various aspects of criminalistics, environmental situations, industry, sports, and racing.

Postmortem toxicology has provided the broadest expertise and accomplishments. Medical examiners and coroners are required to evaluate causes of death resulting from common pharmaceuticals or drugs of abuse. Drug-related deaths cannot be determined by autopsy findings alone since few drugs provide specific lesions. It is, therefore, necessary to identify the presence of the drug in toxicologically significant concentrations since information is needed in conjunction with other investigative and autopsy findings to permit the determination of cause of death. There have been many well-known figures who died of drug overdoses—these include Marilyn Monroe, Elvis Presley, and Lenny Bias. Such highly publicized cases have placed the greatest demands on toxicologists to answer not only the primary question such as cause of death, but also to utilize their expertise in developing other information such as the time and manner of drug ingestion. Today many societal problems deal with a variety of drugs of abuse such as heroin and cocaine. The forensic toxicologist, thus, also serves as a guardian of public health by identifying particular drug problems of society, estimating their magnitude, and identifying unusual situations. This was illustrated by recent epidemics of fentanyl-related

To determine the nature and extent of chemical involvement in a potential human poisoning by:

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1. Isolating and identifying specific chemical substances in appropriate biological or nonbiological specimens using competent techniques
2. Determining the absence of other potentially related chemicals in those specimens
3. Measuring quantitatively any significant identifiable chemical substances found in the specimens
4. Using corroborative tests where necessary to confirm the identity and amount of any substance found
5. Issuing a report of the analytical findings giving information as to the sensitivity, specificity, and reproducibility of the tests used
6. Maintaining appropriate security measures during the collection, storage, and analysis of specimens to guarantee freedom from contamination or spoilage

and by:

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7. Interpreting, when experience allows, the results of an analysis in terms of the effect of the substance(s) found on the behavior and state of health of an individual
8. Supplying information, when possible, regarding the dosage of the chemical ingested, the interval since ingestion, the route and frequency of administration, and other factors pertaining to the exposure
9. Expressing a conclusion, based on the analytical results, as to whether the results are consistent with the history of the incident as known to the toxicologist

Figure 1. Objectives of forensic toxicology as an applied science. (From: Cravey, R. H. and Baselt, R. C. *The Science of Forensic Toxicology*. In: *Introduction to Forensic Toxicology*, eds. R. H. Cravey and R. C. Baselt. Biomedical Publications, 1981.)

deaths in New York and Baltimore resulting from the routine use of heroin. Overall, the postmortem forensic toxicologist determines the presence and/or absence of drugs and metabolites and other chemicals in human biofluids and tissues and evaluates their role as a determinate or contributing factor in the cause and manner of death.

In human performance toxicology, the issues are driving under the influence of alcohol and drugs in a variety of legal settings. Here the toxicologist determines the presence or absence of ethanol and other drugs in blood, breath, and other specimens, and evaluates their role in modifying human performance and behavior. In such situations, toxicologists provide information directly to courts which determine whether specific charges against an individual are warranted at a particular concentration or level of behavior modification.

Forensic urine drug testing for workplace purposes became widespread following the 1986 Executive Order by President Reagan directing a plan of drug testing in the federal workplace. Here toxicologists search for the presence or absence of drugs and metabolites in urine to determine prior use or abuse of drugs. This serves a deterrent function; however, since the rights of individuals and their ability to secure and maintain employment is in jeopardy, the highest concern is raised about the security, testing, and reliability of such analysis.

Toxicologists are also called upon to perform testing of nonbiological specimens. This may involve identification of confiscated drugs of abuse, determination of the composition of pharmaceutical products looking for evidence of tampering, and the determination of environmental chemicals in cases of environmental exposure and contamination.

Forensic toxicology has also been applied to sports, both in humans and animals. Routine use of a wide variety of performance-altering drugs and strength enhancers, such as steroids, has gained prominence in various Olympic and international sports events. Doping of race horses and greyhounds has also occurred. Again, the forensic nature of such testing is paramount since earnings and prizes are at stake.

ORGANIZATIONS IN FORENSIC TOXICOLOGY

Forensic toxicology, as other disciplines, is well recognized by a small group of organizations which have grown very significantly over the last 10 years. Toxicology is represented by the Toxicology Section of the American Academy of Forensic Sciences (AAFS). The AAFS was established in 1948 and has 10 sections focusing on different disciplines, with toxicology the third largest. The Society of Forensic Toxicologists (SOFT), established somewhat later in 1970, is unidisciplinary representing only the interests of forensic toxicologists. SOFT was instrumental in the development of the American Board of Forensic Toxicology (ABFT) and has developed positions on a variety of critical contemporary issues. The International Association of Forensic Toxicologists claims many toxicologists from all countries including the United States. All of these organizations have annual meetings and provide a forum for discussion in the field.

A proliferation of textbooks and scientific literature surrounding this field has developed over the last 20 years or more. A fundamental reference text by Stewart and Stolman was published in 1960 (Stewart and Stolman 1960). Clarke's *Isolation and Identification of Drugs*, originally published in 1969 (Moffat 1986), has seen several revisions. *Methodology for Analytical Toxicology* by Sunshine (1975) developed from prior volumes and *Introduction to Forensic Toxicology* by Cravey and Baselt appeared in 1981 (Cravey and Baselt 1981). Interpretative texts include Baselt and Cravey's *Disposition of Toxic Drugs and Chemicals in Man* (1989), Goodman and Gilman's *The Pharmacological Basis of Therapeutics* (Goodman *et al.* 1990), and Ellenhorn and Barceloux's *Medical Toxicology* (1984). Prominent journals reflecting articles in forensic toxicology include: the *Journal of Forensic Sciences*, the *Journal of Analytical Toxicology*, and the *Forensic Science Review*. The expertise and need for forensic toxicologists has resulted in the publication of a variety of directories of experts. Such are included in the *National Forensic Center Directory of Experts*, a *Listing in Forensic Sciences* by Matthew Bender, and more recently many directories of experts on various computer bases developed by a number of legal and other scientific organizations.

CERTIFICATION IN FORENSIC TOXICOLOGY

Personnel

A number of certification tracks are available in the field of forensic toxicology. The most prominent organization being the ABFT which was established in 1975 (see Figure 2). It offers certification to individuals with Ph.D. and M.S. degrees. Diplomates require a Ph.D. degree in an appropriate natural science, 3 years of postdoctoral experience in forensic toxicology, and must successfully complete a written examination. Diplomates are requalified on a regular basis (presently every 5 years) by providing information describing each individual's current activities.

The ABFT has recently provided for certification of individuals not possessing the doctoral degree. The Forensic Toxicology Specialist of the ABFT is a certification available to individuals with a master's degree and 6 years of appropriate experience. The process involves credentialing by review of experience, transcripts, and recommendations, and successfully passing an examination currently made available by the National Registry in Clinical Chemistry (NRCC). The NRCC provides a specialty examination in toxicology, including forensic toxicology. The NRCC also certifies individuals in toxicology who have a bachelor's degree and appropriate experience. Such individuals must also complete a written examination.

Together, the NRCC and the ABFT, provide

BACKGROUND, FUNCTIONS, AND PURPOSES OF THE AMERICAN BOARD OF FORENSIC TOXICOLOGY, INC.

The need to identify forensic scientists qualified unequivocally who can provide essential professional services for the Nation's judicial and executive branches of government has been long recognized. In response to the professional need, the American Board of Forensic Toxicology (ABFT) was organized in 1975 to provide, in the interest of the public and the advancement of the sciences, a certification program in forensic toxicology. In purpose, function, and organization, the ABFT is analogous to the certifying board in various medical specialties and scientific fields.

The objective of the board is to establish, enhance, and revise as necessary, standards of qualification for those who practice forensic toxicology, and to certify as qualified specialists those voluntary applicants who comply with the requirements of the board. In this way, the board aims to establish a practical and equitable system of rapidly identifying those who possess the requisite qualifications and competence in forensic toxicology.

Certification is based upon the candidates personal and professional record of education and training, experience, achievement, and a formal written examination.

The board is a nonprofit organization incorporated in the District of Columbia. Its sponsors are the American Academy of Forensic Sciences, the Society of Forensic Toxicologists, the California Association of Toxicologists, the Canadian Society of Forensic Sciences, and the Southwestern Association of Toxicologists. The board is composed of officers and other directors, who serve staggered terms and are elected from among nominees designated by the sponsoring organizations, or serve at-large.

Figure 2. Description of American Board of Forensic Technology. (From American Board of Forensic Technology Announcement, 1991.)

certification for individuals with bachelor's, master's, and doctoral degrees; hence, the three-tier approach allows all qualified individuals to gain certification at an acceptable level.

There are other organizations that provide certification. These include the American Board of Clinical Chemistry (Toxicology), which provides certification for Clinical Toxicologists, and the American Board of Criminalistics (ABC), which provides certification for criminalists. Such certification may include toxicologists if toxicologists also meet the basic requirements of the ABC. The American Board of Toxicology provides certification for individuals working in various aspects of experimental toxicology.

Laboratories

Laboratory certification in forensic toxicology has been limited. Some states require specific certification in this field and provide specific requirements. The American Society of Crime Laboratory Directors certifies crime laboratories and some forensic toxicology laboratories would be eligible for this certification.

The Laboratory Guidelines Committee, a joint project of the AAFS, Toxicology Section, and the SOFT, have recently developed a proposal for laboratory certification in forensic toxicology. This currently includes a self-examination checklist and in the future may expand on-site inspection and certification.

The Substance Abuse and Mental Health Services Administration (SAMSHA, formerly NIDA, HHS) provides certification for laboratories engaged in urine drug testing. This certification deals with five specific classes of analytes only. It is associated with proficiency testing at the rate of 6 cycles per year and includes inspection of the laboratory by three qualified inspectors every 6 months. Laboratories certified by HHS are permitted to test specimens from federal employees and for appropriate federal drugs of abuse programs.

The College of American Pathologists (CAP) also provides a certification program in forensic urine drug testing. Their program is similar to HHS's, however, it includes additional drug classes, requires 4 cycles of proficiency testing each year, and a single annual on-site inspection coupled with an intermediary 6-month self-inspection. The CAP program has recently been expanded to include laboratories performing steroid testing.

STANDARDS OF PRACTICE

The various practices of forensic toxicology are not highly standardized. Activities of forensic toxicology laboratories are often driven by the laws in the various jurisdictions in which they operate. Since the laboratory work generally provides evidence for ultimate use in court proceedings, the requirements of various courts and case law often dictate such practices. Most forensic toxicology laboratories are contained in government settings and may not be regulated at all; many states do not regulate these laboratories, while several such as New York, California, and some others, have very specific requirements. The interest in clinical laboratory testing and the recent passage of the Clinical Laboratory Improvements Act controls laboratory testing in the private sector and may include some aspects of laboratory practices in the government sector. As time goes on, considering the legal implications and challenges, as well as the enhancements in technologies, communications, and concerns for the public, greater regulation would be expected.

The forensic toxicology organizations have been quite concerned about laboratory standards of practice. Jointly, the AAFS, Toxicology Section, and the SOFT have developed laboratory guidelines which have been accepted by both organizations (see Figure 3). This represents a first attempt at developing reasonable expectations which can be applied across various jurisdiction lines. These guidelines were published in 1991 and include a preamble and 9 sections along with comments, an appendix, and a self-evaluation checklist.

The standard of practice is also dictated by various consensus statements issued by these organizations. The AAFS has issued position statements dealing with GC/MS confirmation criteria as well as the need for confirmation in urine/drug testing (see Figure 4). The SOFT has issued statements dealing with issues in hair testing entitled "Consensus Opinion Summarizing the Current Applicability of Hair Analysis to Testing for Drugs of Abuse" which was revisited and revised in 1992. SAMSHA (HHS) convened a consensus conference in 1989 looking at the various aspects of the practice of workplace urine drug testing. Designed for the federal sector, HHS certification represents the most stringent certification for any aspect of forensic toxicology testing and has become a de facto standard for the relevant industry.

FORENSIC TOXICOLOGY LABORATORY GUIDELINES

PREAMBLE

In response to the Guidelines for Federal Workplace Drug Testing Programs recently issued by the U. S. Department of Health and Human Services, the Society of Forensic Toxicologists and the Toxicology Section of the American Academy of Forensic Sciences appointed a joint committee of members to recommend a supplementary set of guidelines for the practice of forensic toxicology. The federal guidelines, especially with respect to laboratory personnel and operating procedures, may not always be appropriate for other types of forensic toxicology, and the guidelines set forth below represent recommendations of the Society/Academy committee to respond to that issue. These suggestions do not necessarily reflect our opinions about the minimum requirement for any laboratory, and have no regulatory purpose; rather, they are intended to assist laboratories engaged in the practice of forensic toxicology in achieving future goals.

The committee has concluded that specific guidelines for the practice of forensic toxicology would be appropriate for three defined areas:

Postmortem Forensic Toxicology, which determines the absence or presence of drugs and their metabolites, chemicals such as ethanol and other volatile substances, carbon monoxide and other gases, metals, and other toxic chemicals in human fluids and tissues, and evaluates their role as a determinant or contributory factor in the cause and manner of death;

Human-Performance Forensic Toxicology, which determines the absence or presence of ethanol and other drugs and chemicals in blood, breath, or other appropriate specimen(s), and evaluates their role in modifying human performance or behavior. (The analysis of ethanol in breath, although important, was not considered by the committee because such tests are not conducted in a laboratory setting); and

Forensic Urine Drug Testing, which determines the absence or presence of drugs and their metabolites in urine to demonstrate prior use or abuse. (Because this subject has been covered by the Department of Health and Human Services Guidelines and by the College of American Pathologists Accreditation Program, it was not discussed further by the committee and will not be discussed in this document.)

The specific aims of the committee, with respect to postmortem and human-performance forensic toxicology, have been to provide detailed guidelines for laboratory practices and to prepare a checklist for self-evaluation that may also serve as an important component of a program designed to prepare a laboratory for accreditation. The committee unanimously agreed that a secondary aim of its deliberation should be to develop a voluntary accreditation program for laboratories performing postmortem and human-performance toxicology.

SECTIONS

Operating Procedures

Personnel

Samples and Receiving

Security and Chain of Custody

Analytical Procedures

Review of Data

Quality Assurance

Reporting of Results

Safety

Comments

Appendix

Self-Evaluation Checklist

Figure 3. Forensic toxicology laboratory guidelines of the American Academy of Forensic Sciences, Toxicology Section, and the Society of Forensic Toxicologists, 1991.

**AMERICAN ACADEMY OF FORENSIC SCIENCES, TOXICOLOGY SECTION
POLICY STATEMENT
REQUIREMENTS FOR CONFIRMATORY TESTING**

Confirmation of results is essential in forensic toxicology. Positive results of toxicological screening tests, regardless of the method used, and positive toxicological analysis results obtained by immunoassay methods should either be adequately confirmed before the results are used for forensic purposes or be clearly designated as "unconfirmed" results.

Analysis methods used for attempted confirmation of presumptive results must be appropriately sensitive and specific or unequivocally selective for the analyte(s) in question and must be based upon different chemical or physical principles than the initial analysis method(s).

Figure 4. Policy statement of the American Academy of Forensic Sciences, Toxicology Section, 1986.

Standardized methods are often considered and discussed. The forensic toxicology community, although recognizing the need for standardization and uniformity of results, has resisted standardized methods. Such methods are regularly employed by OSHA and as such have greatly restricted development of new methodologies. The field of forensic toxicology has grown, changed, and advanced so rapidly that it would be hindered by standardized methods, at least at this time.

EDUCATION AND TRAINING

Forensic toxicology is an applied discipline; hence, forensic toxicologists generally hybridize from other areas of science. There are few specific programs designed to train forensic toxicologists. Historically, specific training has been limited to four institutions. At the University of Maryland, in cooperation with the Medical Examiner's Office, forensic toxicologists have been trained initially by Dr. Henry Friemuth and more recently by Dr. Yale H. Caplan. Other programs include the University of Indiana with Dr. Robert Forney, Sr.; Duquesne University with Dr. Charles Winek; and St. Johns University with Drs. Jess Bidanset and Joseph Balkon. These represent the major attempts at training moderate numbers of forensic toxicologists. A number of other associations of a more limited and less comprehensive nature have existed as well. In addition, a number of toxicologists have been trained in job-related applied programs. These include individuals who already had received M.S. and Ph.D. degrees, but came to work either at some institutions in a postdoctoral or research associate capacity and then went on to fulfill a

role as a forensic toxicologist. Many such individuals were trained by Dr. Irving Sunshine in Cleveland and Drs. Friemuth and Caplan in Baltimore. Others continue to do this and most toxicologists practicing today at the Senior or Director level have migrated from a number of key institutions and were effectively trained on the job. There exists a great need for future training in forensic toxicology at all levels.

RESEARCH IN FORENSIC TOXICOLOGY AND DRUG ABUSE

Research in forensic toxicology has generally been limited in the past to adaptations of practical situations; however, in recent times the enhanced awareness of drug abuse as a major problem in society has increased interest and support for research. The research has focused in several areas.

Diagnosis of Drug-Related Toxicity and Death

Here focus is generally on correlation of postmortem findings with drug concentrations in relevant specimens. Situations in which death has been clearly established as a result of the drug ingestion must be compared to situations in which the drug is present, but the cause of death is clearly established by other means. Unlike the clinical situation where therapeutic drugs are given and repeated specimens may be obtained in individuals under observation, the postmortem situation is limited to specimens taken at one point in time and must consider the effects of decomposition and postmortem redistribution. Such studies focus on establishing maximum limits which would be consistent

with therapeutic use and minimum limits which would be considered sufficient for toxic manifestations (fatality). This work is exemplified by a variety of publications over the last several years including the following dissertation subjects:

- The Microdetermination of Cyanide in Fire Fatalities (Roy E. Altman, Jr., Ph.D.)
- Chemical Diagnosis of Methadone-Related Death (Bernard C. Thompson, Ph.D.)
- Tricyclic Antidepressant Drug Fatalities: The Correlation of an Experimental Model of Cardiotoxicity with Medical Examiner Cases (John H. Hebb, Jr., Ph.D.)
- Phencyclidine (PCP) Interaction with Ethyl Alcohol and Morphine: Pharmacology and Disposition of PCP and Metabolites in Rats and Human Postmortem Specimens (Donna Bush, Ph.D.)
- Distribution of Amitriptyline and Other Tricyclic Antidepressant Drugs in the Postmortem Interval: *In Vitro* Cellular Factors, An *In Vivo* Rabbit Model and Human Medical Examiner Cases (Anthony Costantino, Ph.D.)
- The Role of Ecgonine Methyl Ester in the Interpretation of Cocaine Concentrations in Postmortem Blood (Daniel S. Isenschmid, Ph.D.)
- Measurement and Interpretation of Heroin, 6-Acetylmorphine, and Morphine Concentrations in Biological Tissues Obtained From Heroin Users and Heroin-Related Deaths (Bruce A. Goldberger, Ph.D.)

Population and Trend Studies

Much research in forensic toxicology has focused on the identification and documentation of the incidence of drug use, abuse, and deaths. Such research is critical for identifying problems in society and developing means for appropriate countermeasure.

Measures of Intoxication and Impairment

Although much research has been conducted on

ethyl alcohol as a model for studies dealing with the effects of intoxicating chemicals on the human body, the alcohol model is uniquely limited. It is often important in forensic proceedings to establish a degree of intoxication or impairment. Physical signs of impairment often do not correlate with concentration. Further, it is difficult to obtain objective evidence of clinical impairment in many situations; hence, law enforcement depends upon the utilization of concentrations of alcohol and drugs in biological fluids and tissues. Research in this area includes studies dealing with concentrations and levels of impairment in treated subjects, as well as the development of models to determine drug impairment. It is clear at this time, as exemplified by the publication of the consensus paper "Drug Concentrations And Driving Impairment" (Blanke *et al.* 1985) that establishing specific impairment concentrations for drugs without objective clinical evidence is difficult. Research continues developing such correlations.

Drug Abuse

Interest has been renewed in mechanisms and diagnosis of drug use and abuse. Focused in a limited number of centers, such as NIDA's Addiction Research Center in Baltimore, Maryland, illicit drugs are administered to clinical subjects, who are tested for response and drug concentrations.

LEGAL APPLICATIONS

Forensic toxicology requires the general acceptability of scientific principles as related to any applicable branch of science. In addition, forensic science requires a standard of legal sufficiency. The proper evaluation of each testing principle to ensure its utility with a high degree of certainty is essential. Hence, most new scientific principles undergo excessive scrutiny, including meeting the standard described as the Frye Test. Forensic toxicology must also meet the standards of accuracy and integrity required for expert testimony. Not only must the scientific principles be legally sufficient, but the individuals conducting the test must demonstrate a high degree of scientific capability, training, and understanding to be admitted as an expert witness. The expert is unique in the judicial system since a properly qualified expert witness is the only witness allowed to offer opinions while all other witnesses must limit their testimony to facts. The legal application of forensic toxicology also encompasses the interpretation

of results. This is an additional function of the expert witness who must rely on basic experiences, plus the consensus of opinions. Such consensus is difficult to reach in the relevant scientific community, but is facilitated by publications and consultation with peers at scientific meetings.

CHALLENGES FOR THE FUTURE

Future challenges for forensic toxicology may deal with the specimen, the analysis, the instrumentation, and the processes. Choice of specimen, that is the proper specimen for the test, and the increased ability to test for more substances in more specimens utilizing smaller volumes will increase the capability to render reliable interpretations. The interrelationship of various specimens in multiple specimen analysis will facilitate greater understanding of the toxic nature of many substances. Analysis will continue to improve with increased application of both chemical and chromatographic principles. New adaptations of existing technology, as well as the development of newer refined technology, will increase the ability to deal with more and smaller specimens. Instrumentation continues to be reduced in size and portability as well as cost. Such modifications will increase the comprehension of testing that can be accomplished. Processes, that is the nature of analysis, the mixture of techniques, and/or the sequencing of events will be facilitated by automation, robotics, and other more sophisticated devices. These will enable tests to be done more efficiently, perhaps with fewer personnel. The limited personnel available in the field of forensic toxicology limits the amount of work that can be done. Hence, automation can be looked at to enhance testing, thus facilitating evaluation and interpretation.

THE PAST AS PROLOGUE

A number of quotations and statements from textbooks are offered to illustrate the development of ideals of the profession. Many, although proposed many years ago, are quite true today and forecast the future. It is merely the means and the application of the means to the end that have changed.

"Some poisons may act on one important organ only ... but much more commonly they act on several organs at once; and the action of some of them is complicated in an extreme degree."

Sir Robert Christison, *A Treatise on Poisons*, 1845

"There is scarcely a part of the body that may not be examined with profit."

Walter S. Haines, *A Text-Book of Legal Medicine and Toxicology*, 1904

"I shall ... endeavor to show how powerful an instrument a medicolegal investigation may become in skillful hands."

Sir Robert Christison, *A Treatise on Poisons*, 1845

"When you have excluded the impossible, whatever remains, however improbable, must be the truth."

Sherlock Holmes, in A.C. Doyle's *The Adventure of the Beryl Coronet*

"Toxicology is still in the middle of the third day of creation. The seeds have been planted and some vegetation has been brought forth but much more is yet to come."

I. Sunshine, *Toxicology*, 1978

"Forensic toxicology was not created by Arthur Conan Doyle, and it did not diminish in importance with the retirement of Sherlock Holmes, but these two individuals are probably preeminent in people's minds when the topic is discussed. Nevertheless, forensic toxicology remains an important specialty within the field of toxicology. Despite the misconception that the forensic toxicologist toils in the nether world of the dissecting room amidst moldering bones and decaying corpses, the public at large depends on his talents. This does not reduce the significance of other specialties in toxicology; it does point up the need for other specialists to recognize when they are functioning as forensic toxicologists. Perhaps it would be wise to admit not only that forensic toxicology is a specific discipline, but that all toxicologists may eventually function in the area of forensic science.

Forensic toxicology as it exists today can be said to have fulfilled its commitment, and there is no doubt that it will continue to be in demand in the future. However, the profession must remain malleable and responsive to the needs of our rapidly changing society in order to meet the challenges that lie ahead."

Vincent Lynch, *Current Status of the Profession, Introduction to Forensic Toxicology*

"On the bright side, we predict that forensic toxicologists, using advanced analytical techniques, will

become much more adept at gathering analytical data on complex chemical substances and their metabolites. They will use this data in increasingly sophisticated ways to answer the numerous questions that always surround a human poisoning incident. They will expand their horizons from investigation of sudden death to problems of acute and chronic exposure to toxic substances in the home, the workplace, and the external environment. The forensic toxicologist is first and foremost an analyst, and his unique skills will always have extensive applicability in our chemical world."

R. H. Cravey and R. C. Baselt, The Future of Forensic Toxicology, *Introduction to Forensic Toxicology*

These quotations have brought us through time to today, but are indicative of a continuing future. We may reach the day when we look at multiple mass spectrometers operated by a robotic wrist computer configured to produce the sensitivity of one atom. We may be able to test anything, but will always need the toxicologist to interpret and testify.

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Section II

EXTENDED ABSTRACTS

Ion Trap GC/MS Confirmation in Urine Drug Testing

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Urine drug testing involves a two-step process:

- 1) Screening
- 2) Confirmation of Positives

The "Gold Standard" in confirmation is Gas Chromatography/Mass Spectrometry (GC/MS). The use of the Ion Trap 800 Mass Spectrometer (Finnigan MAT, San Jose, CA) allows collection of a full mass spectrum for each material tested due to the increased sensitivity over other mass spectrometers. Full spectral data is the most conclusive as to the identification of drugs and their metabolites compared to just monitoring a few ions. Confirmation of drugs using this technology is used in our laboratory. Derivatization is an important tool in toxicology as many of the metabolites are too polar to chromatograph properly. Two reagents have been used to a large extent in our laboratory:

- 1) N-Methyl-Trimethylsilyl-Trifluoroacetamide (MSTFA)
Pierce Chemical Co., Rockford, IL (Cat. No. 48911)
- 2) N-Methyl-bis-Trifluoroacetamide (MBTFA)
Pierce Chemical Co., Rockford, IL (Cat. No. 49701)

MSTFA is used with the marijuana metabolite (9-carboxytetrahydrocannabinol) and the cocaine metabolite (benzoylecgonine). MBTFA is used with the various amphetamines which are encountered in drug testing some of which are controlled and some of which are over-the-counter medication. These procedures have both been designed to minimize sample handling.

MATERIALS AND METHODS

1) MSTFA

- a) Marijuana metabolite (Fredrick and Fowler 1985)

Five milliliters (ml) of urine are added to a 15 ml glass screw cap tube containing 1 ml of 11.5N KOH. The tube is capped and rotated at room temperature for 30 - 45 minutes. Then sufficient 6N HCl is added to make the pH <2 (acidic) and the resulting solution is extracted with 3 ml of solvent (360 ml of hexane and 40 ml of ethyl acetate) by rotation for 45 minutes. The mixture is centrifuged and the upper (organic) layer is added to an aluminum evaporation cup (Toxi-lab®, Inc., Irvine, CA) containing a paper disc. After evaporation, the disc is transferred to a small vial containing a glass insert. Ten µl of MSTFA is added to the insert and heated for 30 minutes at 75°C. Six µl of MSTFA is added to the tube and 31 µl is injected into the GC/MS (Ion Trap) coupled to a HP 5890 gas chromatograph (Hewlett-Packard, San Fernando, CA). The fused silica capillary column is a 15 meter 0.25 mm OD and 0.1 µ film thickness (Quadrex Corp., New Haven, CT) and temperature programed from 75°C to 300°C at a 15 /min rate with a initial hold at 75°C for 4 minutes. The derivatized metabolite elutes at 1050 seconds. Three major ions 488, 473, and 371 are easily searched after a full spectrum is collected.

- b) Cocaine (Taylor *et al.* 1987)

Extraction of 5 ml of urine in a 7 ml capped tube (Dyana Tek Industries, Lenexa, KS, Cat. No. 7-39A) with 2 ml of a chloroform:isopropanol mixture (9:1) for 30 minutes by rotation yields an organic extract which contains benzoylecgonine. This extract is placed

in an aluminum evaporation cup containing a fiber disc. Concentration at room temperature produces a disc which is placed in a small capped vial containing a glass insert. Ten μl of MSTFA are added to the tube and heated at 70°C for 45 minutes. The addition of 6 μl of MSTFA after heating and injection of 3 μl into the GC/MS using the above conditions elutes the derivatized benzoylecgonine at 800 seconds. Three major ions 362, 240, and 82 can be searched from the full scan mass spectrum.

2) MBTFA

a) Amphetamines (Brettell 1983)

Sympathomimetic amines are extracted from basic urine ($\text{pH} = 9$) using an organic solvent. The same procedure as above (7 ml capped tube, 5 ml of urine, 2 ml of organic solvent extraction, [Sood 1992] rotation for 30 minutes, centrifugation, and evaporation in an aluminum cup containing a fiber disc) produces the disc containing the desired amphetamine. The disc is treated with three drops of acetonitrile. One half μl of MBTFA and three μl of the acetonitrile injected onto the column produces the derivatized amphetamine. This "on-column" procedure leaves the disc intact for additional analysis such as thin layer chromatography. The same GC/MS column conditions are used on all toxicological analysis.

RESULTS AND CONCLUSIONS

These procedures described here have been in use in our laboratory for the past 3 years and have allowed us to confirm a large number of samples at or near the NIDA cut-off levels using full spectral data. At present, we are enrolled in three proficiency testing programs (AACC/CAP, Toxi-Lab, and State of Pennsylvania). The very important confirmation of amphetamine has not been a problem with the MBTFA on column derivatization procedure. These procedures require a minimum of sample handling and analyst time. At present, we are looking at solid phase extraction (SPE) and autosampling as a means of increasing sample throughput.

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(THC) 11-nor- Δ^9 tetrahydrocannabinol metabolite

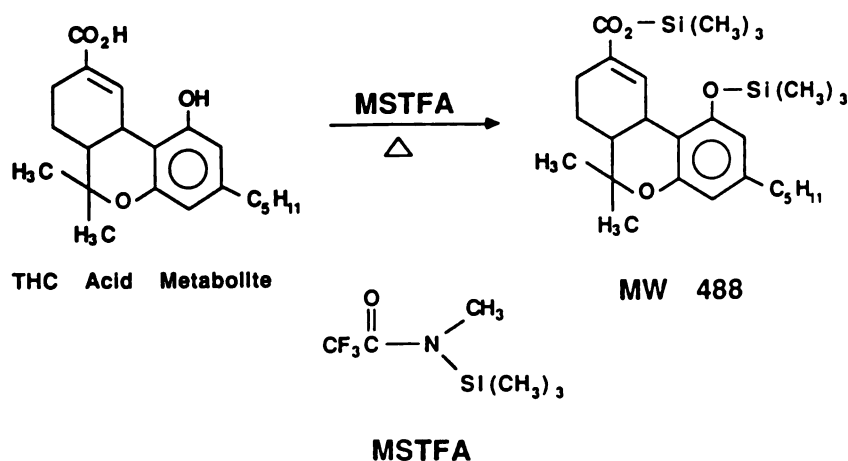


Figure 1. Derivatization Reaction of Marijuana Metabolite with MSTFA.

COCAINE

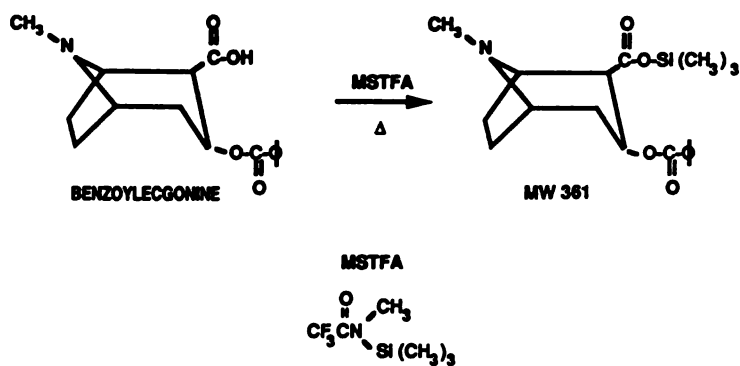


Figure 2. Derivatization Reaction of Benzoyllecgonine with MSTFA.

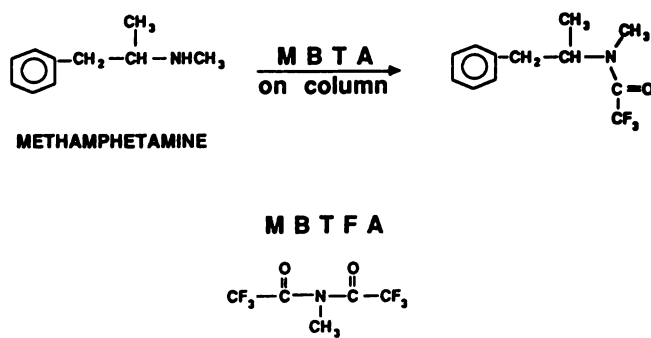


Figure 3. Derivatization Reaction of Methamphetamine with MBTFA.

AMINE IDENTIFICATION USING M B T F A

AMINE		MOLECULAR ION
	AMPHETAMINE	232
	METHAMPHETAMINE	246
	PHEENTERAMINE	246
	PHENYLPROPANOLAMINE	230
	EPHEDRINE	244

Table 1. Molecular Ion Formed by "ON Column" Reaction with MBTFA.

Evaluation of a New Concept of Quality Control for Drugs of Abuse in Urine

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The quality-control concept of clinical chemistry is always dedicated to the analysis of one substance in one analyte. This analyte is usually serum or plasma. Records documents have to include:

- Precision of the method used
- At least one standard and one reference sample
- Contained in one run
- Control limits
- Qualitative chemical analysis checked daily with reference samples

But for toxicological analysis, it is important to add:

- Limits of detection (LOD)

In accordance with the rules of the IFCC, precision, accuracy, and specificity are tested in this procedure. In analytical toxicology, however, we have the problem that these conditions have to be fulfilled not only for one single substance, but for several substances in body fluids (serum, whole blood, urine, or stomach content). We use a limited number of methods for toxicological analysis, but we have a large variety of possible substances. So it seems suitable, not to practice substance-related quality control, but system

or instrument-specific and method-specific quality control procedures.

We want to discuss a model which demonstrates this principle on the GC/MS analysis for drugs of abuse in urine for the following reasons:

- These procedures are firm to mandatory guidelines of the NIDA in the USA
- The analytical instrumentation is rather uniform
- Many optimal internal standards in form of the deuterated drugs are easily available
- The number of substances to be looked for is reasonably limited

The procedure starts with a system-specific or instrument-specific quality control:

A methanolic mixture of the deuterated standards (20 ng) of the following drugs were analyzed by GC/MS. Some results are summarized in Table I.

TESTS FOR REPRODUCIBILITY, FOR LIMITS OF DETECTION, AND FOR SPECIFICITY

The LODs from a urine sample for the drugs mentioned below have been obtained. They are better than the NIDA limits in the confirmation test.

Table 1				
Substances	RI	C _v %	PA x 10 ³	C _v %
Amphetamine	1212	<0.2	1.022	7.2
Methamphetamine	1346	0.1	0.306	14.6
Phencyclidine	1941	<0.1	1.054	3.9
Benzoylcegonine	2158	<0.1	1.240	10.3
Morphine	2331	<0.1	2.052	5.9
Codeine	2363	<0.1	0.367	7.3
THC-COOH	2397	<0.1	0.226	6.1

ANALYTICAL PROCEDURE

One ml-spiked urine is mixed with phosphate buffer pH 6 and passed through a Bond Elut Certify column (from Analytichem International/Varian). Acids and bases are eluted from the column after rinsing in the usual manner.

Deuterated internal standards are added after elution, evaporated to dryness under nitrogen and derivatized with 100 µl PFPA and 70 µl PFPOH.

After evaporation to dryness under nitrogen, an ethyl acetate solution is injected in the GC/MS (HP 5890 with MSD).

GC conditions: PhMe silicone 5% 12 m x 0,2 x 0,33 µm (Ultra 2 from HP).

CONCLUSIONS

1. The quality control procedure in urinalysis by GC/MS is used as an example to demonstrate the concept of a possible system-specific and method-specific program.
2. The system-specific procedure allows to control the precision of the measuring process, due to instrument-related errors and bias, and the specificity.
3. The method-specific control manages the bias in extraction, the control of accuracy, specificity, recovery, and the limit of detection.
4. The concept can be extended to other drugs, drug metabolites and poisons which are of interest in forensic and clinical toxicology.

Impairment Levels of Triazolam

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Triazolam, a low-dose benzodiazepine, is the most widely prescribed hypnotic used in Canada, with an estimated 30 million prescriptions filled in the period 1979 to 1990 (Neutel *et al.* 1991). It is encountered in "driving under the influence of drug" type cases both alone, and in combination with other depressant agents, by the R.C.M.P. Forensic Laboratories in Canada (Joynt 1991).

The objectives of this study were: (1) To demonstrate the effects of triazolam on a small group of healthy volunteers; (2) To determine whether there is a specific minimal blood level of triazolam, associated with impairment; and (3) To determine the whole blood to plasma ratios of triazolam in paired samples.

The study protocol involved 5 normal subjects, employees of our laboratory, each receiving a 0.5 mg oral dose of triazolam (Halcion®) at about 0900 hours. The subjects (2 male, 3 female) subjectively rated their own status of impairment, and were objectively rated by a judge on a 5-point scale of impairment. Whole blood samples were drawn at 3 separate points: that time when they felt some effects, but still felt capable of operating a motor vehicle ("some effects"); that time when they were definitely impaired to the point of unsafe operation of a motor vehicle ("impaired"); and a final sample was drawn sometime later, when some improvement was noted ("post impairment").

Triazolam was quantified in whole blood and paired plasma by methodology routine to our laboratory. One-ml samples were spiked with an internal standard (1.16 ng of nimetazepam), basified with 20 µl-concentrated NH₄OH, and extracted with 5-ml n-butyl chloride by gentle inversion for 10 minutes.

The samples were centrifuged at 3000 rpm and 10°C for 15 minutes. The n-butyl chloride was removed and evaporated to dryness with N₂ gas. The residues were dissolved in 15 µl ethyl acetate and analyzed on a

HP5730 gas chromatograph, equipped with a 15-meter DB-17 megabore (0.545 mm) column of 1 micron film thickness. The gas chromatograph was equipped with a Ni63 detector. The oven temperature program was, 220° for 2 minutes, then raised at 8°/min, to 280°, where it was held for 16 minutes. Typical retention times were nimetazepam 10.33 minutes and triazolam 18.09 minutes.

The results of this experiment were:

- 1) One subject of the five studied here did not reach a state of impairment either by subjective or objective rating. That individual did experience "some effects", but at a time much later (106 minutes) than did the remaining four subjects (mean 23 minutes). That individual's blood level at 106 minutes was 4.2 ng/ml, and at 157 minutes was 3.6 ng/ml, which is consistent with normal absorption. That individual seemed to be insensitive to the effects of triazolam.
- 2) The time for onset of "some effects" in the remaining four subjects were 25, 25, 22, and 20 minutes. The blood levels accompanying those times were 2.0, 2.0, 4.2, and 4.4 ng/ml, respectively.
- 3) The time required to reach "impaired" ratings of the remaining 4 subjects were 70, 40, 57, and 52 minutes, and the associated blood levels were 4.3, 4.4, 4.6, and 8.3 ng/ml, respectively.
- 4) The subject who attained an "impaired" level of 8.3 ng/ml was clearly the most impaired of all the subjects, both by subjective and objective assessment.

- 5) Thirteen pairs of data were available from matched blood and plasma samples. Whole blood to plasma ratios ranged from 0.74 to 1.13, with a mean of 0.93.

In conclusion, we note that one subject, or 20% of our population, did not reach an "impaired" condition with 0.5 mg triazolam. The remaining 4 subjects reached an "impaired" state, associated with blood levels of 4.3, 4.4, 4.6, and 8.3 ng/ml (mean 5.4 ng/ml). In light of these values, it may be reasonable and conservative to suggest that impairment occurs at blood levels of 5 - 6 ng/ml and greater, in the majority of subjects.

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Comparison of Roadside Breath-Alcohol Analysis with Subsequent Analysis in Relating Evidentiary Results Back to the Time of Driving

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Many jurisdictions have driving while intoxicated (DWI) legislation requiring the establishment of 0.10 g/210L or more at the time of driving. Evidentiary breath-alcohol analysis always incorporates a time delay from offense to analysis for several reasons. Expert forensic witnesses are then frequently asked to estimate the breath-alcohol concentration (BrAC) at the time of driving through retrograde extrapolation which raises a host of assumptions and uncertainties. The present study was designed to evaluate the expected change in an individual's BrAC from the time of driving to later evidential analysis and assess the estimation reliability of evidential analysis.

MATERIALS AND METHODS

Individuals arrested for DWI ($n = 79$) were administered roadside Pre-Arrest Breath Tests (PBT) employing the Alco-Sensor III (Intoximeters, Inc., St. Louis, MO) by trained police officers. They subsequently provided duplicate evidential breath alcohol analyses employing the BAC Verifier DataMaster (National Patent Analytical Systems, Inc., Mansfield, OH). Following evidential analyses they again submitted to a single breath analysis employing again the PBT instrument. All data was truncated to two digits.

Differences for each analytical method (PBT and evidential) were then evaluated and compared statistically. The variances for the two methods were also compared with the variance ratio F test. In addition,

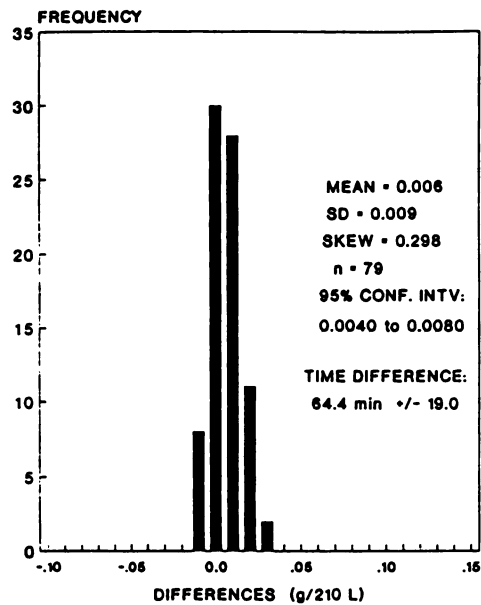
the differences were plotted against their means to assess trends with respect to concentration (Altman and Bland 1983).

RESULTS AND DISCUSSION

The mean time between the duplicate PBT analyses was $X = 64$ minute with span of 35 to 120 minutes. The PBT differences were: $X = 0.006$ g/210L with a 95% confidence interval of 0.004 to 0.008 g/210L. The evidential analysis differences were: $X = -0.001$ g/210L with a 95% confidence interval of -0.0035 to 0.0015 g/210L. Figure 1 shows the histograms of the 2 difference distributions. Figure 2 shows the plots of difference against means for the 2 methods with no significant trends indicated. The variance ratio F test resulted in $F = 1.49$, $p = 0.024$.

The duplicate PBT data shows a slight but significant decreasing trend in BrAC from roadside to subsequent analysis. The variability associated with the PBT distribution is comparable to the evidential distribution and suggests that individuals do not change systematically in their blood-alcohol concentration due to metabolism or adsorption. Further, this study better addresses the change in BrAC over time by employing the same instrument (PBT) and is therefore more informative than previously reported (Gullberg 1991). Subsequent evidential analysis performed in duplicate are good estimates of BrAC at the time of driving and systematic changes due to adsorption or metabolism are not expected to exceed the total method variability (Dubowski 1988).

P B T Differences



Evidential Differences

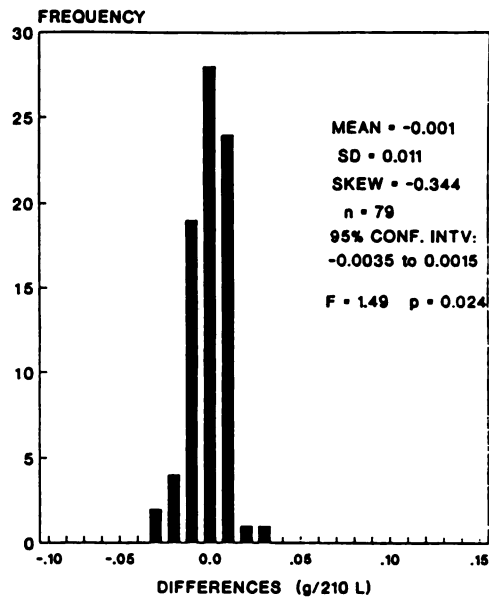
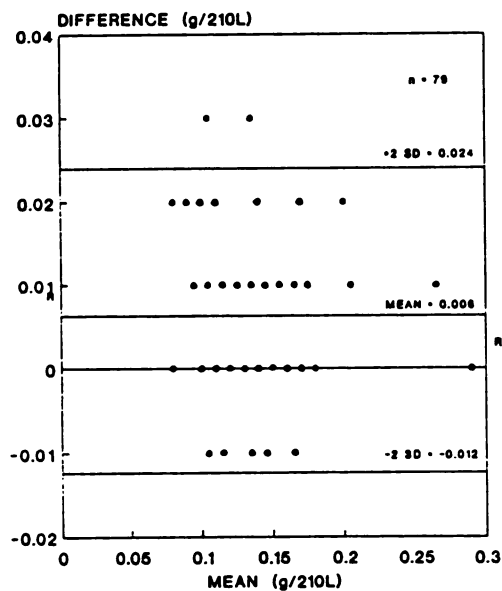


Figure 1. Distribution of duplicate differences for PBT and evidential analyses.

P B T Differences



Evidential Differences

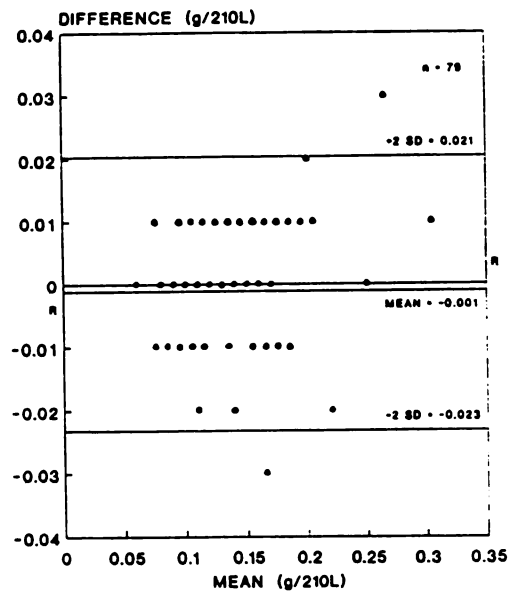


Figure 2. Plot of duplicate differences against their means for PBT and evidential analyses.

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Robotic Method for the Analysis of Morphine and Codeine in Urine

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The abuse of the opiates, notably heroin, remains a major drug problem affecting all sectors of society. The number of opiate-related cases received at this crime laboratory continues to increase. Accurate, rapid, and cost-effective methods of analysis are necessary in order to produce reliable results in an efficient and timely manner, as required by the legal system. Thus, an automated analytical procedure, utilizing a Zymark robotics system (Zymark Corporation, Hopkinton, MA) was developed which could analyze the high volume of samples requiring confirmatory testing for opiates.

A Zymate Laboratory Automation System was used for the hydrolysis, solid phase extraction, and derivatization of samples prior to gas chromatograph/mass spectrometer (GC/MS) (Hewlett Packard, Palo Alto, CA) analysis. This procedure was a modification of a robotic method for cocaine analysis by Taylor and Le (1991). The robotic system consisted of the various extraction stations secured to sections of wedge-shaped aluminum platforms. These mounted stations, or PySections, are located in a circular pattern around the Zymate II robot. Two mL of urine sample is poured by the analyst into 16 x 100 mm disposable test tubes and placed into the temperature-controllable sample rack. Further handling of the sample through the hydrolysis incubation, extraction, and derivatization procedure was performed robotically. The sample aliquot, the addition of the internal standard, and any dilutions were verified by weight. Glucuronidase enzyme (Sigma Chemical Company, St. Louis, MO) was added and the sample

incubated for 4 hours. The pH was adjusted to 8.8 with 5% NaOH and then extracted by solid phase extraction. The sample, after evaporation, was reconstituted with bistrimethylsilyl trifluoroacetamide + 1% trimethylchlorosilane and acetonitrile and placed into the derivatization station for 20 minutes at 70°C. After derivatization, the sample was injected into a GC/MS operating in the selective ion monitoring mode.

The precision of pipetting and dispensing of the sample aliquot, internal standard, diluent, and diluted sample was monitored by weighing. The overall precision of each transfer was excellent with coefficient of variations (CV) ranging from 0.4% to 1.8%. The within-run and between-run precision was also monitored and was found to have acceptable CV's. The recovery of morphine was determined to be 80% - 87%.

The Zymate Laboratory Automation System has enabled the testing for morphine and codeine to be fully automated. In addition to saving man-hour expenditures, automation decreases human exposure to hazardous chemicals and specimens. The Zymate robotic system has demonstrated that it is a reliable, precise, and cost-effective alternative to manual techniques.

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Differentiation of Unusual Phenethylamine Derivatives Using Thin Layer Chromatography

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In the forensic setting, many controlled and uncontrolled phenethylamine derivatives are often encountered as substitutes for methamphetamine and amphetamine (1, 2, 3, 4, 5, 8, 12, 14, 15, 17). Traditional color and crystal tests are not well documented for many of these derivatives. Because of similarities in structure, gas chromatography retention times are very close and sometimes indistinguishable. For the same reasons, mass spectral data can be so similar, that interpretation is difficult. To improve chromatography and facilitate interpretation of mass spectra, some manipulation of the sample is required (*i.e.*, derivatization). Because GC/MS is complex, time-consuming, and requires highly trained personnel, it is more appropriate as a confirmation method.

In this report, thin layer chromatography (TOXI-LAB™, TOXI-LAB, Inc., Irvine, CA) is used as a screening method to quickly and easily differentiate several phenethylamine derivatives from one another. This is accomplished using a TLC technique with a 5-parameter specificity (color reactions with 4 color reagents and R_f). The solvent system used for separation was: ethyl acetate; methanol; acetaldehyde; water (87:3:0.2:1.5 by volume) plus 5 μ L of concentrated ammonium hydroxide per milliliter of solvent. Chromatograms were developed in 3 mL of developing solvent to 10 cm. The solvent was removed from the chromatograms by heating on a hot plate at 70°C for about 30 seconds or until dry. The main detection system for broad-spectrum screening consisted of dipping the chromatograms into several reagents sequentially, and observing the results at four stages (Michaud and Jones 1980). At each stage the

chromatograms were removed from the reagents and viewed while still wet. Following a 5 to 15 minute exposure to formaldehyde vapors (10 to 15 mL of 37% formaldehyde in a stand-off jar), the chromatograms were then dipped slowly into concentrated sulfuric acid. The chromatograms were viewed after about 20 seconds to 1 minute (Stage I). Next, they were dipped quickly into and out of water (Stage II). At Stage II the colors appeared immediately for some drugs; others appeared within 10 seconds. The chromatograms were viewed subsequently under long wave (366 nm) ultraviolet light (Stage III). At this stage many of the drugs showed an immediate absorbance or fluorescence. At Stage IV, the chromatograms were dipped into modified Dragendorff's reagent. Various shades of brown were visible immediately (O'Brien *et al.* 1982). Other screening methods, such as immunoassay, do not have tests available for these unusual phenethylamine derivatives. The method described provides a rapid screening method, not only for urine samples but also for the analysis of solid-dose narcotics suspected of containing these derivatives.

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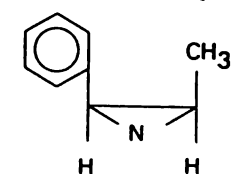
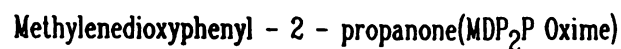
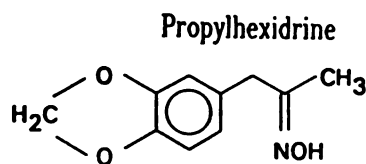
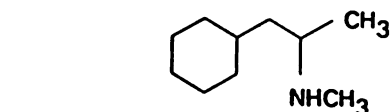
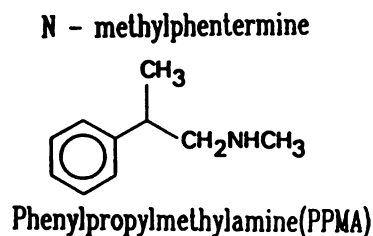
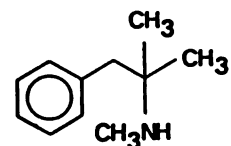
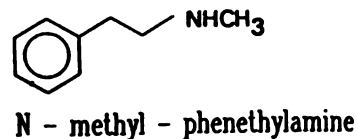
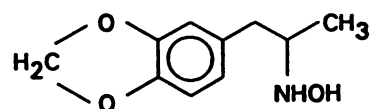
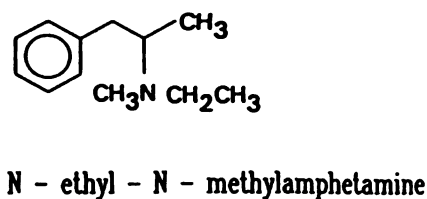
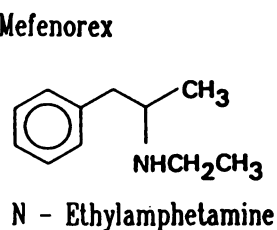
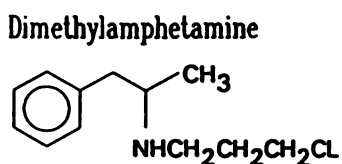
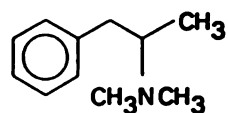
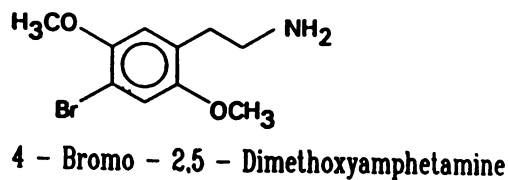
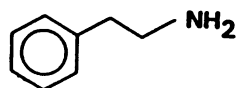
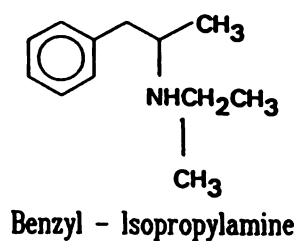


Figure 1. Structures of phenethylamine derivatives.

Methodology for the Simultaneous Quantitation of MDA, MDMA, and MDE Utilizing Solid Phase Extraction (SPE) and Gas Chromatography/Mass Spectrometry (GC/MS)

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As the use of CNS stimulants increased, methods were developed in the laboratory to better detect and quantitate these substances from suspected abusers. With the onset of "designer drug" use, a rise in amphetamine analog [MDA (methylenedioxy-amphetamine), MDMA (Methylenedioxy-methamphetamine), and MDE (methylenedioxyethyl-amphetamine)] abuse necessitated the development of a method to both detect and quantitate these drugs from biological specimens.

SPE techniques have been researched and proven effective in the extraction of this class of drugs. The following method provides an efficient and timely means of obtaining an analysis-ready sample for confirmation by GC/MS.

The specimen is prepared by adding both an internal standard (IS) and 1 M phosphate buffer, pH 9.1 and vortexing. After an SPE column has been conditioned with methanol and 1 M phosphate buffer, pH 9.1 (7% isopropanol), the specimen is added and drawn through the column under low vacuum (<2" of mercury). Subsequently, 1 M phosphate buffer, pH 9.1 is added, followed by drying under high vacuum (>15" of mercury). Sample collection is achieved by elution with an acetonitrile-n-butyl chloride mixture to

which two drops of 10% hydrochloric acid (HCl) in methanol is added. Upon drying, derivatization of the sample proceeds with ethyl acetate and heptafluorobutyric anhydride (HTFBA) being added, and incubation of the sample at high temperature for 30 minutes. The sample is then dried, reconstituted with ethyl acetate, and placed in an autosampler vial for injection onto a GC/MS. A Hewlett-Packard 5890 GC and 5970 MSD in selective ion monitoring (SIM) mode with Targetc autoquantitation software is used in this procedure. Ions used in detection and quantitation are as follows: MDA - 135, 240, 375; MDMA - 254, 210, 389; MDE - 268, 240, 403; N-Propylamphetamine (IS) - 282, 240, 91. Quantitation is based on a calibration curve utilizing three standard samples of known concentration. Two quality controls, one high and one low, are also included.

Successful results have been obtained in several criminal cases where initial positive amphetamine screening had led to a negative amphetamine/methamphetamine confirmation. Upon reanalysis using the subject methodology, both MDA and MDMA were then detected and quantitated. As both MDA and MDMA are controlled substances, the benefit of this procedure is self-evident.

Field Performance of Current-Generation Evidential Breath-Alcohol Analyzers

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We evaluated the field performance in law enforcement use of the Model 5000-D Intoxilyzer (CMI, Inc., Owensboro, KY), a current-generation evidential breath-alcohol analyzer using infrared spectrometry (Dubowski 1992). Reported here are the accuracy of analysis results as measured by concurrent vapor-alcohol control analyses (Dubowski and Essary 1991) with 34°C TOXITEST II simulators (CMI, Inc., Owensboro, KY) and the precision of results as measured by duplicate breath-alcohol analyses on separate breath specimens.

MATERIALS AND METHODS

Data collected remotely from six instruments at separate sites by periodic downloading with an ADAMS computer program (CMI, Inc., Owensboro, KY) were combined, and analyzed by computerized standard statistical techniques (Natrella 1963). Our data base encompasses the results of alcohol analysis on paired, consecutive breath specimens from 740 subjects, and 779 control vapor-alcohol analyses accompanying each subject test or performed at regular equipment maintenance intervals after the earlier of 25 tests or 30 days. Data analysis included analysis of variance (ANOVA), linear regression, paired-t tests, and relative and cumulative distribution of differences between paired subject test results and between paired simulator test target values and control test results.

VAPOR-ALCOHOL ANALYSIS RESULTS

The experiment signed and absolute differences between simulator target value and control test results are summarized in Table I, with their descriptive population statistics. Figures 1 and 2 show the relative

frequency distribution and the cumulative frequency distribution, respectively, of these paired absolute vapor-alcohol concentration differences.

DUPLICATE BREATH-ALCOHOL ANALYSIS RESULTS

The experiment signed and absolute differences between paired breath-alcohol test results ($BrAC_1$ - $BrAC_2$) are summarized in Table II, with their descriptive population statistics. Figures 3 and 4 show the relative frequency distribution and the cumulative frequency distribution, respectively, of these paired absolute breath-alcohol concentration differences.

We conclude that field performance of these current-generation evidential breath-alcohol analyzers and simulators corresponds closely to that achieved in our controlled laboratory studies, and fully meets established standards and operational requirements for traffic law enforcement and similar applications.

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Table 1. Paired Sample Difference [#] for Simulator Alcohol Control Test Results, g/210 L.									
Target Concentration	Number of Tests	Signed Difference				Absolute Difference			
		Mean	SD of Mean	Mode	Experimental Span	Mean	SD of Mean	Mode	Experimental Span
0.06	21	-0.0035	0.0018	-0.004	-0.009 to -0.001	0.0035	0.0018	0.004	0 to 0.009
0.08	49	-0.0001	0.0032	-0.002	-0.009 to +0.005	0.0025	0.0018	0.002	0 to 0.009
0.09	183	-0.0008	0.0026	-0.001	-0.008 to +0.008	0.0021	0.0017	0.001	0 to 0.008
0.10	306	-0.0004	0.0027	0	-0.010 to +0.006	0.0021	0.0017	0.001	0 to 0.010
0.11	71	0	0.0017	0	-0.003 to +0.007	0.0012	0.0012	0.001	0 to 0.007
0.12	149	0.0016	0.0044	0.004	-0.010 to +0.010	0.0039	0.0026	0.001	0 to 0.010
ALL	779	-0.0001	0.0032	0	-0.010 to +0.010	0.0024	0.0020	0.001	0 to 0.010
[#] Control Target Concentration Minus Result.									

Table 2. Paired Sample Difference [#] Between Duplicate Breath-Alcohol Analysis Results, g/210 L.								
Number of Tests	Signed Difference				Absolute Difference			
	Mean	SD of Mean	Mode	Experimental Span	Mean	SD of Mean	Mode	Experimental Span
740	0.0005	0.0094	0.001	-0.030 to 0.033	0.0069	0.0064	0.001	0 to 0.033
[#] BrAC ₁ Minus BrAC ₂								

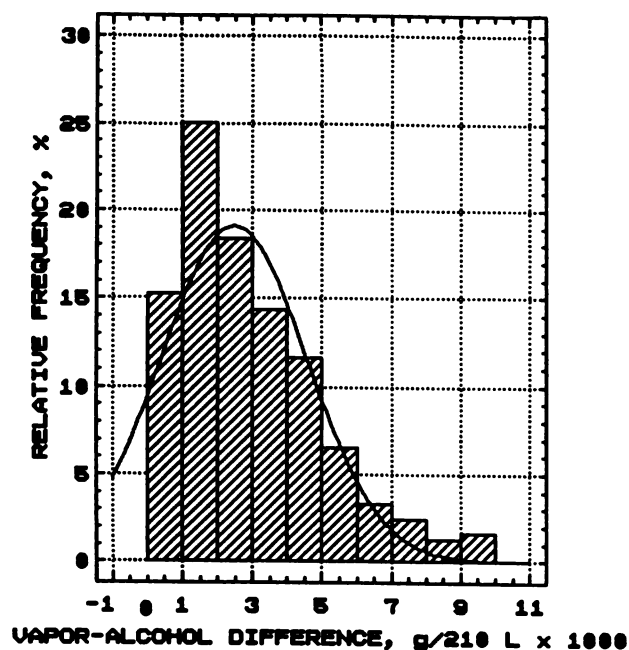


Figure 1. Relative Frequency Distribution of Absolute Vapor-Alcohol Concentration Differences (Target Value Minus Control Test Result).

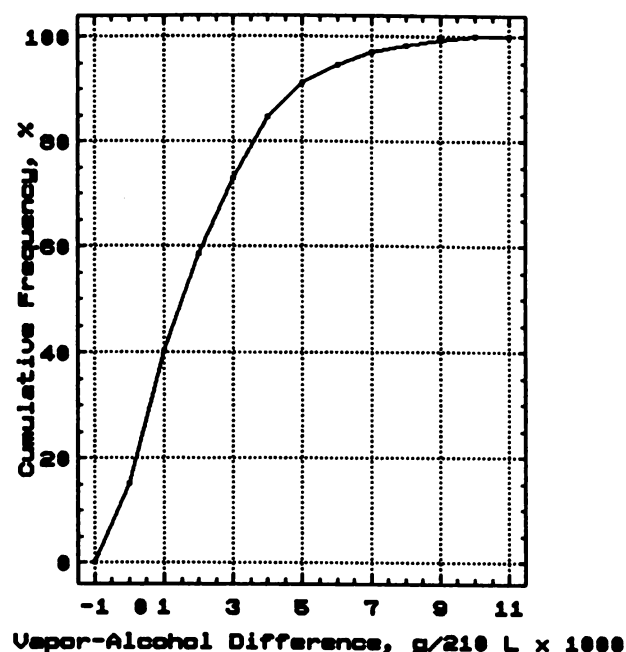


Figure 2. Cumulative Frequency Distribution of Absolute Vapor-Alcohol Concentration Differences (Target Value Minus Control Test Result).

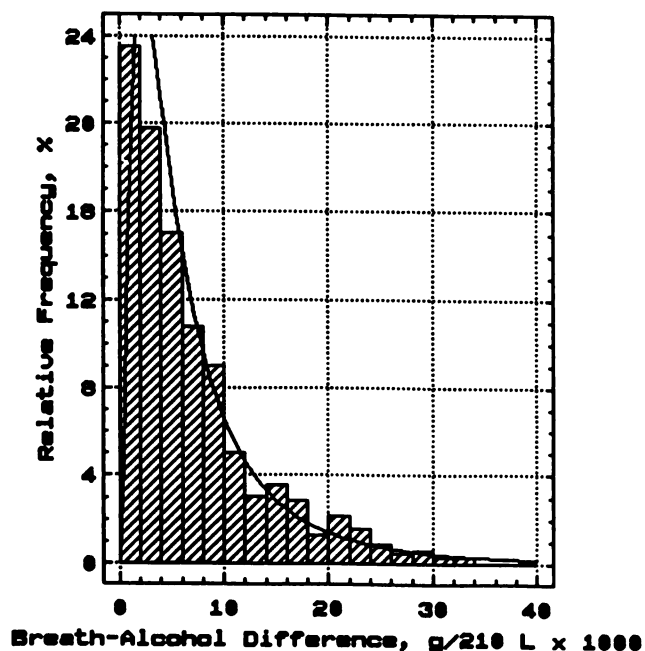


Figure 3. Relative Frequency Distribution of Absolute Breath-Alcohol Concentration Differences ($BrAC_i - BrAC_j$).

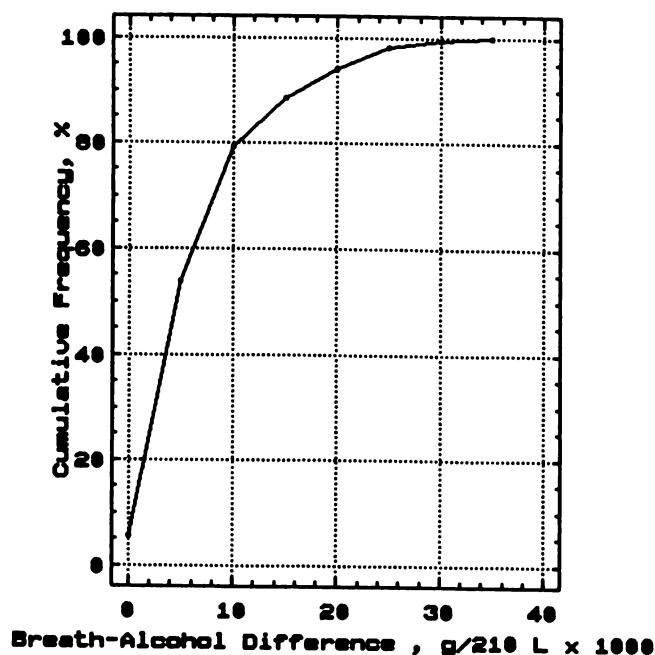


Figure 4. Cumulative Frequency Distribution of Absolute Breath-Alcohol Concentration Differences ($BrAC_i - BrAC_j$).

Involvement of Alcohol and Drugs in Driving Incidents: A Comparison of Nonfatal and Fatal Incidents

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The involvement of alcohol and/or drugs in causing or contributing to driving incidents has long been postulated. Some of these driving incidents lead to the driver being arrested by police and charged with driving while intoxicated (DWI). Other incidents result in injuries or fatalities. Both types of incidents will be covered in this presentation.

Analytical methods used included headspace gas chromatography for alcohol (Foerster and Garriott 1981) and solvent extraction followed by gas chromatography for other drugs (Foerster *et al.* 1978; 1979). Gas chromatography/mass spectrometry was used when necessary to confirm identification of various drugs.

During 1991, blood samples from 2,503 cases of DWI were submitted to our laboratory for analysis for alcohol, drugs, or both. Of these, only 343 (13.7%) had a blood alcohol concentration (BAC) less than 0.11%. And of these, only 54 (2.2%) were negative for both alcohol and drugs. The average BAC for all cases was 0.179%. Analyses for drugs were conducted in 624 cases and were positive in 59 cases, with 74 substances being identified. Most prevalent was cocaine, with diazepam second. Our laboratory does not conduct analyses for tetrahydrocannabinol.

Traffic fatalities can involve the driver of the vehicle, a passenger, or another person struck by the

vehicle. During 1991, 400 such fatalities were investigated by our office, with 195 being drivers, 110 being passengers, and 95 not being within the car. Of the drivers, 88 were positive for ethanol; the average BAC for drivers testing positive was 0.150%. Sixty-six were positive for drugs (most prevalent were diazepam/nordiazepam and cocaine). Fifty drivers had neither alcohol nor detectable drugs.

A greater proportion of drivers stopped by police were positive for alcohol (97%) than of drivers killed in a traffic accident (45.1%). However, drugs were detected in a greater proportion of fatally injured drivers (33.8%) than in those drivers stopped by police and tested for drugs (17.1%).

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Studies on the Selection of an Appropriate Marijuana Radioimmunoassay Cutoff Level for Workplace Drug Urinalysis

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Immunoassays may be responsive to compounds of the same category, while gas chromatography/mass spectrometry (GC/MS) is specific for the identification of individual compounds. Current practice in workplace drug testing programs requires GC/MS identification of a targeted drug/metabolite above a cutoff concentration and a prior immunoassay screen test result above an identical or different cutoff level. In light of this requirement, manufacturers frequently reduce the cross-reacting characteristics of immunoassay reagents to improve their correlation with the GC/MS identification of the targeted drug/metabolite. This current study presents empirical data of ABUSCREEN® Radioimmunoassay (AB-RIA) for Cannabinoids (Roche Diagnostic Systems, Branchburg, NJ) manufactured during the period of March 1987 - October 1991 to illustrate the significance of adopting an appropriate cutoff level for an immunoassay used in a workplace drug testing program.

AB RIA is based on competitive binding of ^{125}I -labeled antigen and unlabeled antigen (analyte in the test sample), in proportion to their concentrations, to a limited quantity of antibody in the reaction mixture. As a heterogeneous immunoassay, AB RIA utilizes a second antibody to precipitate the bound antigen, thus separating it from the unbound ones (Figure 1) before measuring radioactivity. In practice, the urine specimen

and ^{125}I -labeled counterpart of the analyte were combined with the first antibody with an APS Pipetting Station (Model 25004, Micromedic Systems, Inc., Horsham, PA). After a 30-minute incubation period, a Micromedic High Speed Dispensing Station was used to add the second antibody. Upon completion of another 30-minute incubation period, the mixture was centrifuged. Tubes were then decanted, drained, and blotted. Pellets containing bound antigen were counted for 0.2 minutes with a Micromedic Apex 10/600 Gamma Counter. Results thus obtained in counts-per-minute were converted to ng/mL using a dose-response curve.

All specimens used in this study were extracted and derivatized for GC/MS analysis using a procedure developed by the U.S. Navy's Drug Screening Laboratories. Briefly, with a deuterated analog as the internal standard, 11-nor- Δ^9 -tetra-hydrocannabinol-9-carboxylic acid (THC-COOH) was extracted with a solid phase extraction approach using the Dupont Prep Type A Extraction Cartridge and processed by the Dupont Prep I Automated Sample Processor (Wilmington, DE). THC-COOH was then alkylated with iodomethane in TMAH/DMSO. A Hewlett-Packard (HP) 5970B mass selective detector (MSD) coupled to a HP 5890A gas chromatograph was used for analysis. A 15-m (0.251 mm ID) J & W DB-5

(0.25 µm film thickness) capillary column (Folsom, CA) was connected to the MSD through a direct capillary interface. The injection port was a capillary split injector with a silanized glass insert. The carrier gas (helium) flow rate was approximately 1.0 mL/min with a split ratio of 10:1. The following ions resulting from electron impact ionization were monitored: *m/z* 372, 357, and 313; and 375 and 360 for THC-COOH and deuterated THC-COOH, respectively. The first ion listed for each compound was used for quantification.

Approximately 195,000 specimens were tested during the March 1988 - February 1992 period. Data were searched (and placed into 4 groups) from a pool of approximately 32,000 specimens that can be definitely identified using the reagent with package inserts dated March 1987, March 1988/November 1988, June 1989/May 1990, and October 1991. (Identical reagent specificity data were provided for the following pairs of package inserts: March 1988/November 1988 and June 1989/May 1990.) RIA data of specimens that are within a narrow protocol dynamic concentration range (30 - 200 ng/mL) are correlated with their corresponding GC/MS results.

Raw data points and their respective linear regression lines of the 4 data groups are shown in Figures 2a-2d. Respective correlation coefficients, linear regression equations, and apparent RIA THC-COOH concentrations equivalent to 15 ng/mL THC-COOH as calculated from the regression equations are tabulated in Table 1. Improved correlations between the RIA and GC/MS results are observed generally in parallel with increasing reagent specificity (Table 2). Realizing that immunoassays are responsive to other cannabinoids/metabolites in the specimen, the observed correlations (0.00 for no correlation; 1.00 for perfect correlation) are significant. The significant reduction of the estimated RIA apparent THC-COOH concentration that corresponds to 15 ng/mL THC-COOH is most informative. (It was noted that selection of data points may alter the numerical values of the regression coefficients somewhat, but not the estimated apparent RIA concentration to a significant extent.) These empirical data suggest that, if the THC-COOH concentration determined by GC/MS is of the primary concern, the RIA cutoff level should be adjusted from approximately 110, 100, 70, then to 50 ng/mL using the reagents described in the package inserts dated March 1987 to October 1991. Screen test reagent specificity data should thus dictate the selection of the cutoff level.

Table 1. Correlation Information of RIA and GC/MS Data.

Reagent Package Insert Date	Test Date	Correlation Coefficient	Correlation Equation ^a	RIA Apparent THC-COOH Equivalent to 15 ng/mL
March 1987	11/09/87 - 01/21/88	0.426	$Y = 85.6 + 1.82 X$	113 ng/mL
March 1988 - November 1988	11/29/88 - 04/24/89	0.405	$Y = 92.4 + 0.65 X$	102 ng/mL
June 1989 - May 1990	06/08/89 - 02/25/90	0.500	$Y = 56.0 + 0.89 X$	69.4 ng/mL
October 1991	10/04/91 - 02/19/92	0.665	$Y = 39.2 + 1.00 X$	54.0 ng/mL
^a X: GC/MS THC-COOH concentration; Y: ABUSCREEN® cannabis metabolites concentration (expressed in THC-COOH equivalent).				

Table 2. Variation of Specification of ABUSCREEN® RIA Kit^a

Compound	Approximate Percent Cross-Reactivity					
	03/87	03/88	11/88	06/89	05/90	10/91
11-nor-8-THC- Δ^9 -carboxylic acid	244	76	76	76	76	49.5
11-Hydroxy- Δ^9 -THC	38	20	20	<5	<5	2.8
Δ^9 -THC	5	3	3	<5	<5	1
8- β -11-Dihydroxy - Δ^9 -THC	11	7	7	<5	<5	1.9
8- α -Hydroxy-cannabinol	<5	13	13	<5	<5	1.4
11-Hydroxy-cannabinol	<5	7	7	<5	<5	<1
Cannabinol	<5	<5	<5	<5	<5	<1
Cannabidiol	<5	<5	<5	<5	<5	<1

^aData taken from package inserts.

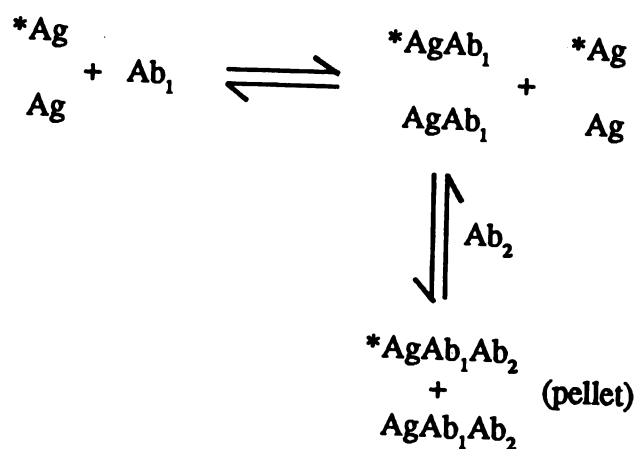


Figure 1. Principle of ABUSCREEN® radioimmunoassay.

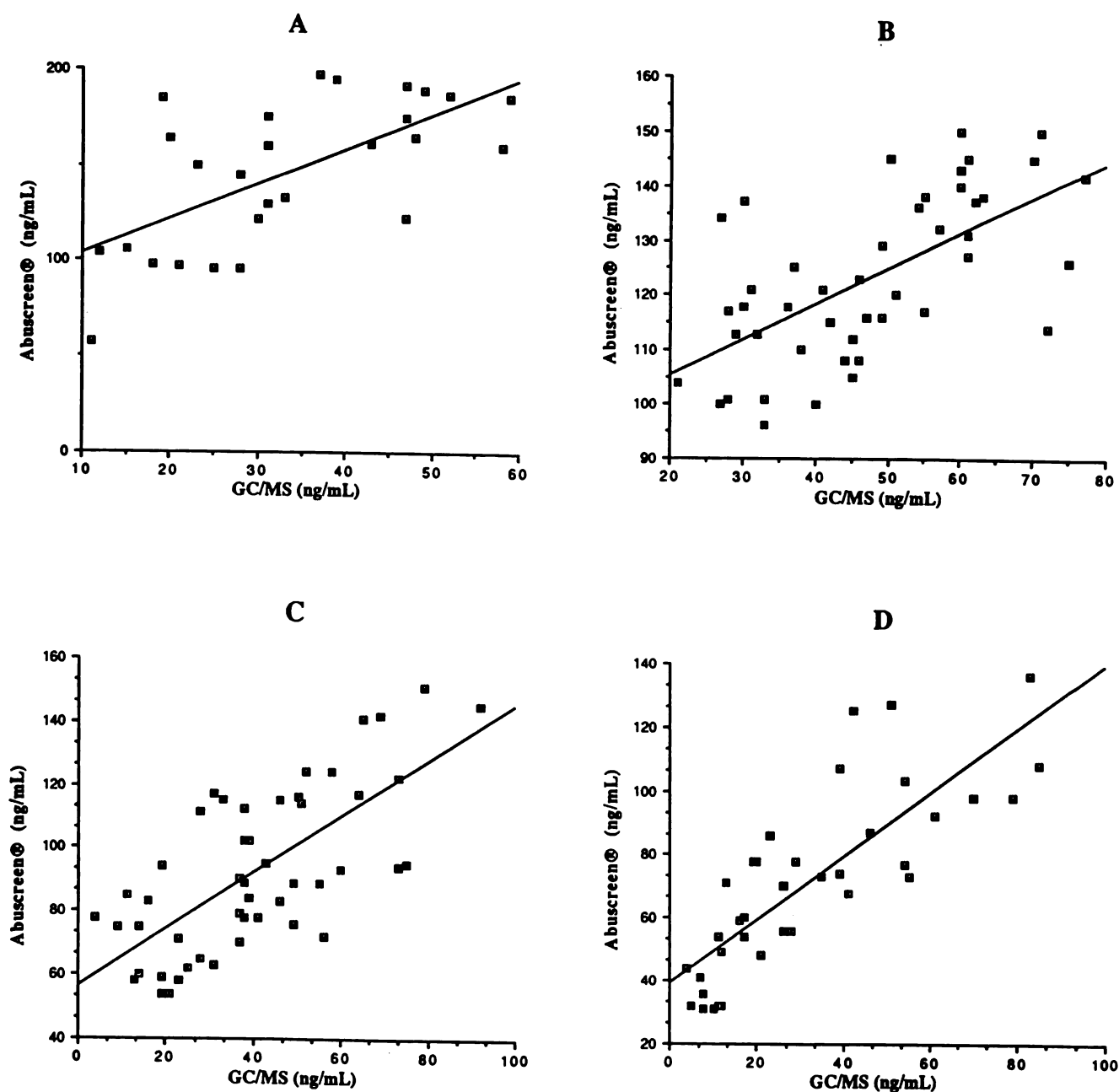


Figure 2. Correlation of GC/MS THC-COOH concentration vs. ABUSCREEN® cannabis metabolites concentration (expressed in THC-COOH equivalent): Data collected during the period of 11/09/87 - 01/21/88 (A); 11/29/88 - 04/24/89 (B); 06/08/89 - 02/25/90 (C); and 10/04/91 - 02/19/92 (D).

Poppy Seed Ingestion: The Oregon Perspective

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Oregon's driving under the influence of intoxicants (DUI) laws clearly describe that operating a motor vehicle while under the influence of alcohol and/or drugs is unlawful. To demonstrate the magnitude of the drug-impaired driver, a study performed by this laboratory showed that of 145 drivers arrested for DUI, 42% were found to be positive for controlled substances (Hayes 1991).

Recent articles indicate that ingestion of poppy seeds can lead to levels of morphine and codeine high enough to cause a false interpretation of a subject's urine. This issue becomes critically important when trying to evaluate the level of impairment of a person operating a motor vehicle (Bjerver *et al.* 1982; Elsohly *et al.* 1988; Fritsch and Prescott 1985; Hayes *et al.* 1987; Pettit *et al.* 1987; Selavka 1991; Struempfer 1987; Zebelman *et al.* 1987).

The National Highway Traffic Safety Administration (NHTSA), along with the Los Angeles Police Department, instituted a DRUG RECOGNITION EVALUATION (DRE) program in 1986. The DRE examiner observes and records five categories of reactions that demonstrate signs of drug use:

- 1) Vital signs (pulse, temperature, and blood pressure)
- 2) Psychophysical response (coordination of mind and body)
- 3) Signs of administration of drugs (injection sites, etc.)
- 4) Eye response (horizontal/vertical gaze nystagmus, eye conveyance, pupil size under varying light intensities)

- 5) Physical and behavioral characteristics (muscle rigidity or flaccidity, hyperactivity, etc) (Oates 1991)

The primary intent of our experiment was not only to determine that the ingestion of poppy seeds produces positive morphine/codeine levels, but to determine whether a trained drug recognition evaluator (DRE) could observe any significant symptoms of impairment.

The DRE results were critical to the success of the experiment. The individuals, in essence, acted as our medical review officers.

MATERIALS AND METHODS

Poppy seeds were purchased from three local stores. The origin of one source was indicated to have been from the San Francisco area. The remaining two sources were unknown. Three poppy seed cakes were prepared so that each volunteer would consume 25 grams of one type of seeds. Each subject consumed the seeds within 1 hour, and was evaluated for opiate symptoms 2 - 6 hours post ingestion.

Urine screening was done in accordance with Syva's ETS methods. Varian's procedure of acid hydrolysis and subsequent extraction through their solid phase extraction tubes prepared the sample for GC/MS confirmation. Confirmation was done on a Hewlett-Packard 5890 GC/MS using a 200 - 270°C program.

RESULTS AND DISCUSSION

Seven volunteers consumed the seeds with the general response of being extremely full.

Two to 6 hours post ingestion, subjects gave

morphine values ranging from 307 ng/ml to 8,940 ng/ml. Each volunteer was subjected to a series of standardized tests to determine impairment. Characteristics of opiate impairment are listed in Table 1.

Based upon pre and postingestion data (refer to Table 2), evaluators observed no significant changes in pupil size, body temperature, blood pressure, pulse rate, inner clock, nystagmus, or typical field sobriety tests.

This data indicates that an individual who consumes large amounts of poppy seeds does not show symptoms of opiate impairment, even though they may have positive urine results.

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Table 1. Drug Category Symptomatology Chart		
Major Indicators	Narcotic Analgesics	
Horizontal nystagmus	Not present	
Vertical nystagmus	Not present	
Lack of convergence	Not present	
Pupil size	Usually constricted	
Reaction to light	Little or no visible reaction	
Pulse rate	Usually below normal	
Blood pressure	Usually below normal	
Body temperature	Usually below normal	
General indicators	Droopy eyelids; drowsiness; depressed reflexes; low, raspy voice; dry mouth; facial itching; euphoria; fresh puncture marks <u>NOTE:</u> Hippus may be evident during withdrawals. Tolerant users exhibit relatively little psychomotor impairment.	
Duration of effects	3 - 6 hours	
Methods of administration	Oral; smoked	
Overdose signs	Shallow, slow breathing; clammy skin; convulsions; coma; possible death	
Normal Ranges		
Blood Pressure	Pulse	Pupil Diameters
120/70 - 140/90	60 - 90	3.0 - 6.5

Table 2. DRE Evaluation from 0- to 6 Hours

	Age	Sex	Weight (Kg)	Seeds consumed (g)/ Body Weight (Kg)	0 Time			+2 Hours Time		+4 Hours Time		+6 Hours Time		+8 Hours Time		+12 Hours Time	DRE Results 2 - 6 Hours Post-injection
					A	B	C	A	B	A	B	A	B	A	B	A	
Cake A																	
NMH	41	M	79.8	.31	-	-	N	+	310	+	1028	+	1308	NS		+	NC
TJB	42	M	85.1	.29	-	-	N	+	2248	+	1950	NS		NS		NS	NC
Cake B																	
KDM	41	M	82.2	.30	-	-	N	+	2110	+	305	+	3250	NS		+	NC
RAK*	44	M	77.9	.16	-	-	N	+	307	+	329	+	305	NS		+	NC
KOH	42	F	66.7	.37	-	-	N	+	898	+	2570	+	2265	NS		+	NC
Cake C																	
CHV	50	M	113.2	.22	-	-	N	+	2305	NS		NS		+	2110	+	NC
GGD	49	M	84.7	.29	-	-	N	+	1120	+	3111	+	8940	NS		+	NC

N = Normal

NC = No Change

NS = No Sample Given

* = Consumed 1/2 of Poppy Seed Amount

A = Emit

B = GC/MS (ng/ml Morphine)

C = DRE Results

NOTE: Codeine was observed in elevated morphine specimens. No quantitative value was determined. Opiate concentration of the seeds was not determined.

Confirmation of Drugs of Abuse in the Blood and Urine of Driving Under the Influence (DUI) Suspects Using Gas-Liquid Chromatography with a Fourier Transform Infrared Detector in Series with a Mass Selective Detector

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The New Jersey State Police Forensic Science Laboratories routinely analyze blood and urine from DUI suspects for drugs of abuse. Historically, the presence of a drug in blood and urine has been confirmed using a variety of techniques which have included thin-layer chromatography, gas chromatography (GC), high-performance liquid chromatography, and gas-chromatography/mass spectrometry. We now report the combination of GC with a Fourier Transform Infrared Detector and Mass-Selective Detector (GC/IRD/MSD) for the confirmation of drugs of abuse and their metabolites in biological fluids.

A few reports have appeared in the literature describing various aspects of the applications of GC/FT-IR to forensic drug analysis (Kempert 1988) including the analysis of benzophenones (Minagawa 1991) and the discrimination of five monomethylated Fentanyl Isomers (Suzuki 1989). GC/IR/MSD has been used for the high-confidence identification of drugs (Duncan and Deutsch 1989) including the differentiation of side chain isomers of ring-substituted amphetamines (Soine *et al.* 1992) and the qualitative and quantitative analysis of amphetamines and related analogues in human urine (Platoff *et al.* 1992).

The goal of our work was not to find a replacement for GC/MS, but to demonstrate that GC/IR/MSD has sufficient sensitivity to characterize drugs of abuse and their metabolites isolated from DUI suspects' urine and blood. And in addition, that the technique can be used routinely in the forensic toxicology laboratory.

MATERIALS AND METHODS

A Hewlett-Packard 5890A Series II Gas Chromatograph with a split/splitless injector was interfaced with an HP-5965A Infrared Spectrophotometer (IRD) with a wide-band (4000-550 cm^{-1}) MCT detector. The IRD was connected in series with an HP-5970 Mass Selective Detector (MSD). A 0.32 mm I.D. x 0.25 M x 0.52 μm HP-5 fused silica capillary column and a 0.32 mm I.D. x 25 m x 1.0 μm DB-1 fused silica capillary column were used in the analyses. The IRD flow cell temperature varied from 240°C to 290°C depending on the temperature program run. The detector resolution was 8 wavenumbers. Sample Interferograms were collected both 20 seconds before and after every peak apex.

Five mL of urine and 3 mL of blood from DUI suspects was extracted using solid phase extraction

cartridges (Worldwide Monitoring, Inc. - CSDAU-131 cartridges, 130 mg) using a 0.1 M phosphate buffer to adjust to pH = 6 and various solvent washes. The extracts were dried under nitrogen and reconstituted with 50 μ L of chloroform/isopropanol (3/1). A 1 μ L sample of the drug extract was injected into the gas chromatograph. The gas chromatographic conditions consisted of Helium carrier gas at 25 kPa head pressure, 1 μ L splitless injection, and an injection port temperature of 250°C. The gas chromatographic oven was programmed from 120°C to 240°C at 30°C/min and then up to 300°C at 10°C/min.

RESULTS AND DISCUSSION

Figure 1A shows the vapor phase infrared transmission spectrum of morphine injected into the combined GC/IRD/MSD system. Morphine was one of the components extracted by solid phase from 5 mL of a DUI suspect's urine. Figure 1B shows the Total Reconstructed Chromatogram (TRC) of this sample. The presence of multiple drugs and metabolites including methadone and its metabolites, codeine, morphine, 0-6-monoacetylmorphine, and acetylcodeine, were detected in this sample using the GC/IRD/MSD. Vapor phase infrared spectra and mass spectra were easily obtained for all of these drugs and metabolites.

Using this system, solid phase extracts of blood (3 mL) and urine (5 mL) samples taken from DUI suspects were analyzed for drugs of abuse and their metabolites. Vapor phase spectra and mass spectra were simultaneously obtained for each component. Sensitivity and recovery of actual case samples was excellent. Spectra of small peaks resulting from metabolites were easily obtained and allowed the differentiation of closely related isomers and metabolites. The TRC showed good correlation with the Total Ion Chromatogram (TIC) of the retention times of the components of the same sample.

Overall GC/IRD/MSD is a reliable and useful technique for the routine analysis of blood and urine samples of DUI suspects for the presence of drugs of abuse and metabolites. The vapor phase spectra of the drugs of abuse examined were unique and reproducible. The analytical method showed sufficient sensitivity and allowed the differentiation of closely related isomers and metabolites.

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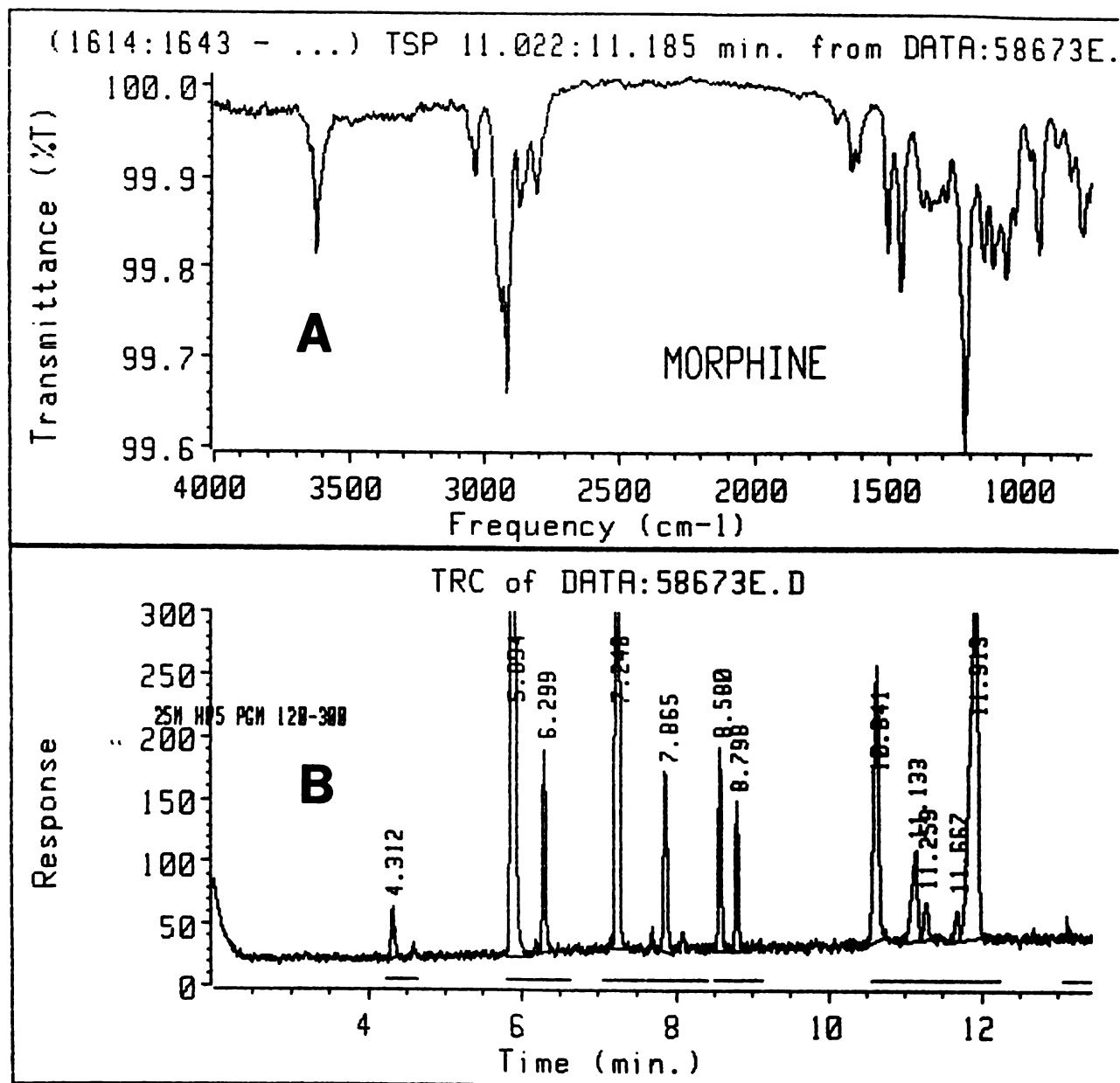


Figure 1. (A) Vapor phase infrared transmission spectrum of morphine injected into the combined GC/IRD/MSD system. Sample was a solid phase extraction (SPE) of a DWI suspect's urine. (B) TRC of SPE from a DWI suspect's urine showing multiple drugs and metabolites.

Agonal Events Leading to Erroneous Conclusions Regarding Antemortem Blood Ethanol Concentrations From Postmortem Blood Samples

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Variations in ethyl alcohol concentration sampled from various sites throughout the body have been observed for many years. Beginning in 1986, a joint study was undertaken by the Toxicology Section of the Palm Beach County Sheriff's Office Crime Laboratory and the District 15 Medical Examiner's Office in Palm Beach County, FL. The purpose of the study was to determine the degree of variability among the sites and then to attempt to identify the causes of the observed differences.

MATERIALS AND METHODS

Three different cardiovascular sites were chosen for the collection of the blood specimens: the right atrium, the lower inferior vena cava, and the ascending aorta. We believe that these sites best reflected the dynamics of alcohol distribution. Blood was collected directly from each site under direct observation with a 14-gauge needle and transferred to 10 mL gray stoppered vacutainer tubes (Becton-Dickinson, Rutherford, NJ). Each tube contained 20 mg of potassium oxalate and 25 mg of sodium fluoride as an anticoagulant and preservative. Ocular fluid was pooled from both eyes and also placed in a gray stoppered tube. Gastric contents were removed and stored in a 50 ml plastic container, unpreserved. All specimens were kept under refrigeration until analysis.

Analysis of the specimens was performed by a modified method of Basalt. The column utilized was a

3 foot, 2 mm id glass column packed with GP 60/80 Carbowax B/5% Carbowax 20 m (Supelco No. 1-1766). The instrumentation was a Hewlett Packard 5890 gas chromatograph utilizing electronic integration with n-propyl alcohol as the internal standard. The direct injection technique was linear from 0.005 to 0.60% ethanol. Oven temperature was maintained isothermal at 85°C, injection port and detector at 200°C. Whole blood (0.2 ml), ocular fluid or gastric contents were diluted with 0.2 ml of the 0.15% n-propyl alcohol internal standard. One microliter of this mixture was then used for analysis.

Previous data had been published and this data is only for the 1991 calendar year.

Of the 776 cases submitted for toxicological analysis during 1991, 29.5% or 229 cases, had ethyl alcohol present. In 53% of those cases, the ethyl alcohol content was equal to or greater than 0.10% in at least one blood site. One-hundred and twelve cases were included in this study in which all five specimens were collected. In 33% of those cases, the alcohol content varied by greater than 20% from the highest to the lowest blood concentrations measured in a single case. Predominant reasons for the variations, as determined from hospital records, accident reports, witness statements, and autopsy findings, were from attempted resuscitative attempts by emergency personnel, agonal aspiration of alcohol-laden vomitus into the bronchial passages, and obvious recent ingestion of alcohol that

had not reached equilibration at the time of death.

Conclusions of this study strongly indicate that relying on a single, and in many cases two sites for testing, yields insufficient data to form a solid opinion

as to the antemortem blood ethanol concentration. Documentation as to antemortem events as well as those findings at autopsy need to be communicated to the individual responsible for the analysis and interpretation of the alcohol results.

Stability of Drugs of Abuse in Urine Samples Stored at -20°C

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Mandatory guidelines issued by the Department of Health and Human Services for federal workplace drug testing programs require that drug testing laboratories retain all confirmed drug-positive urine samples for 1 year in frozen storage during which time retesting may be requested. Therefore, it is important to evaluate the stability of drugs of abuse during such long-term frozen storage. Generally, drug stability studies have been restricted to a few drugs, have been short-term studies, or have not evaluated the effects of frozen storage. Therefore, this study was undertaken to evaluate the stability of common drugs of abuse or their metabolites following 12 months of frozen storage.

We have determined the stability of amphetamine (AMP), benzoylecgonine (BENZ), cocaine (COC), codeine (COD), methamphetamine (MAMP), morphine (MOR), 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (9-COOH-THC), and phencyclidine (PHEN) in 236 physiological urine samples stored at -20°C for 12 months. Presumptive analyses were conducted using the Syva EMIT® reagent system on an Hitachi 717 analyzer and quantitative analyses were conducted gas chromatography/mass spectrometry (GC/MS) with cutoff concentrations of 5 ng/ml for 9-COOH-THC and PHEN and 100 ng/ml for each of the other drugs. Following the initial quantitative analysis, the samples were stored, in the plastic containers in which they were received, at -20°C for 12 months after which the concentrations were determined again. Samples containing drugs at concentrations above the cutoff concentration were identified as positive and those containing drugs at

concentrations below the cutoff concentration were identified as negative.

Generally, a wide range of alterations in drug concentrations was observed following storage. In those samples in which the drug concentrations were above the cutoff concentrations both before and after storage, the ranges and means of the observed concentration changes were: AMP, -35 to +30%, = -3%; BENZ, -56 to 73%, = +10%; COC, -87 to +20%, = -37%; COD, -60 to +252%, = +14%; MAMP, -48 to +25%, = -1%; MOR, -69 to +63%, = +9%; 9-COOH-THC, -70 to +141 %, = -1%; and PHEN, -52 to +11%, = -14%. In 10 samples (AMP, 2; MOR, 3; COD, 1; COC, 4) the drug concentration was less than the cutoff concentration initially and greater than the cutoff concentration at the end of the storage period and in 9 samples (AMP 1; COD, 3 of 38; PHEN 5) the drug concentration was greater than the cutoff concentration initially and less than the cutoff concentration at the end of the storage period. These data demonstrate the difficulty of estimating initial drug concentrations following long-term storage.

Several factors may contribute to the instability of drugs in stored samples. These factors include sample pH, type of storage container, use of preservatives, and analytical variation. In our study, it is reasonable to conclude that variation in the analytical methodology was an important cause of the observed changes in drug concentration especially in those samples with concentration changes of $\pm 25\%$. Future studies should determine the influence of these several factors on the storage stability of drugs.

Assuring Quality of Urine Drug Testing as a Component of a Drug-Free Workplace: The Role of NIDA

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In September of 1986, President Reagan issued an Executive Order requiring all agencies to implement a Drug-Free Federal Workplace. This initiative included drug testing programs for certain designated federal employees and required the Secretary of Health and Human Services to issue comprehensive standards for all aspects of laboratory drug testing, including the accreditation of all laboratories performing urine drug testing services for the federal government. Public Law 100-71, Section 503, further mandated the publication of federal standards for laboratory certification. NIDAL certifies urine drug testing laboratories through the National Laboratory Certification Program (NLCP) to perform testing in accordance with the forensic and analytical requirements published in the Mandatory Guidelines for Federal Workplace Drug Testing Programs (*Federal Register*, Volume 53, Number 69, pp. 11970-11989, April 11, 1988). The guidelines define the primary criteria for laboratory certification to include: (1) adequacy of the laboratory facilities; (2) expertise and experience of laboratory personnel; (3) excellence of the laboratory's quality control program; (4) the performance of the laboratory on any proficiency tests; (5) the laboratory's compliance with standards as reflected in any laboratory

inspection; and (6) any other factors affecting the reliability and accuracy of drug tests and reporting done by the laboratory. The goal of this Drug-Free Federal Workplace Program is drug detection and deterrence of illegal drug use. These guidelines provide that each urine specimen may be tested initially by immunoassay for cannabinoids, benzoylecgonine, opiates, phencyclidine, and amphetamines. Specimens determined to be presumptively positive for these drugs/drug classes by immunoassay must be then analyzed by gas chromatography/mass spectrometry (GC/MS) to confirm the presence and determine the quantity of the drug. In addition to methods of analysis, other essential elements of the laboratory are inspected. These include quality control, standard operating procedures; chain of custody documentation; specimen receiving, preparation, and storage areas; reagents; equipment; and security of the facility. Program actions such as laboratory certification and suspension are based on inspection processes, performance testing, and review of analytical results. The functional relationships of the independent components of this model program which has standardized operations in over 100 forensic laboratories during the past three years will be detailed.

A GC/MS Peak is Described, Which Interferes with the Identification and Quantitation of Morphine

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An unidentified peak on the GC/MS tracing of opiate-positive urine was first observed in January 1991, during the New York City death epidemic associated with fentanyl-laced heroin. Ever since this peak has been seen in a number of opiate positive urine samples submitted for drug analysis, especially along with codeine and morphine, with codeine only, and in specimens where codeine was not detected.

The unknown peak shares at all times the quantitation ion for morphine used in our method (429) and one of the qualifying ions (414), while the other 2 qualifying ions appear to be much less involved (236 and 196). Other specific ions may be present. The main analytical features of this unknown peak are ion ratios well outside the $\pm 20\%$ range of the morphine calibrators and controls, and a slightly longer retention time than morphine. The lengthening of the retention time resulted in shoulders in the morphine peak, or a partially resolved double peak when morphine was present, or independent peak when morphine was not present. The new peak appears 0.03 - 0.05 minutes behind the morphine-d₃ peak, as opposed to the morphine peak which appears within 0.02 minutes. These findings were reproducible upon reextraction and reinjection of the specimens.

Because of the appearance of this peak in many cases the confirmation of morphine in forensic specimens has become problematic even in instances where next to the unknown peak morphine can be unequivocally confirmed. In these cases the relative contribution by morphine to the overall peak morphine is not clear, raising a legitimate doubt whether morphine is definitely present at or above the cutoff concentration. In sum, in our experience in most cases up to one-third of the

specimens submitted for opiates confirmation have to be called negative for morphine.

In this communication we present characteristic features of this unknown entity (henceforth called GSS peak), as well as some of the preliminary attempts made at further defining and researching the nature of the peak, by varying some of the columns and procedures used for the extraction of the opiates from the sample and the concentration of the drug in the sample.

MATERIALS AND METHODS

All chemicals, reagents, enzymes, calibrators, standards, and controls used were of chromatographic purity and highest purity, purchased from commercial sources.

1. Columns

The columns used were purchased from Applied Separations, Inc. (Allentown, PA) and Worldwide Monitoring (Horsham, PA).

2. Instrumentation

HP GC/MS system 5890/5970, with Ultra 1 HP 12.5 m capillary column, cross-linked methylsilicone gum.

3. Solutions

The elution solvent consisted of a mixture of methylene chloride, isopropanol, and NH_4OH (80:20:2.5).

GC/HS PROCEDURES

I. Beta GLUCURONIDASE

- Pour 2.5 ml. urine/standards/controls in screw-cap tubes; add 100 μ l I.S., mix; and add 0.25 ml of beta glucuronidase (8,000 units) 0.13 ml phosphate buffer pH 4.0. 0.1 M.
- Incubate in heating block at 65°C for 1 hour, remove, and cool .
- Adjust to pH 7.0 using 1N acetic acid; centrifuge 15 minutes at high speed.

II. ACID HYDROLYSIS

- Pour 2.5 ml urine/standards/controls in screw-cap tubes; add 100 μ l I.S., mix; then add 0.25 ml 50:50 HCl/H₂SO₄.
- Incubate in heating block at 120°C for 20 minutes, remove, and cool.
- Adjust to pH 7.0 with 0.35 NH₄OH; centrifuge for 15 minutes at high speed.

III. WORLDWIDE EXTRACTION PROCEDURE

- Activation
 1. Add 2 ml of pure CH₃OH , and elute adjusting the vacuum pressure at 3 mm Hg.
 2. Add 2 ml d.i. water, and elute as above.
 3. Add 1 ml phosphate buffer pH 7.0, 0.1 M, and elute as above.
- Specimen Addition and Wash
 1. Add specimens to columns, keeping the vacuum at 3 mm Hg.
 2. Add 2 ml d.i. water, and elute as above.
 3. Add 1 ml phosphate buffer pH 4.0, 0.1 M, and elute as above.

4. Dry for 15 minutes at 20 mm Hg.
5. Add 2 times 1 ml CH₃OH, and elute as above.
6. Dry for 40 minutes at 20 mm Hg.

• Elution and Derivatization

1. Add 2 ml elution solvent to each column and let it drip into assigned screw-cap tubes placed underneath at 3 mm Hg. Increase pressure to 20 mm Hg for 2 minutes.
2. Dry eluate at 40°C under N₂ and reconstitute specimens with 150 μ l ethylacetate.
3. Add 50 μ l BSTFA + 1% TMCS to the tubes, mix, and place the tubes into heating block at 75°C for 25 minutes.
4. Cool and transfer to GC/MS vials.

IV. APPLIED SEPARATION EXTRACTION PROCEDURE

- Activation
 1. Add 2 ml pure CH₃OH, and elute adjusting the vacuum pressure at 3 mm Hg.
 2. Add 2 ml d.i. water, and elute as above.
 3. Add 1 ml 1N acetic acid, and elute as above.
- Specimen Addition and Wash
 1. Add specimens to columns, keeping the vacuum at 3 mm Hg.
 2. Add 2 ml d.i. water, and elute keeping the pressure at 3 mm Hg.
 3. Add 1 ml 1N acetic acid, and elute as above.
 4. Dry for 15 minutes at 20 mm Hg.

5. Add 2x 1 ml CH₃OH, and elute as above.
6. Dry for 40 minutes at 20 mm Hg.
7. Elute, reconstitute, and derivatize as above.

RESULTS

I. Early characteristics of the GSS peak.

Early findings involving the GSS peak were obtained with acid extraction and using Applied Separation columns. Below in Table 1 are the results obtained in that early stage of our observations.

Table 1. Ion Ratios (IR) and Retention Times (RT) of the GSS Peak, Where Morphine Was Not Observed.

IR414/429	.26 +/- .06 (SD) N=14
236/429	.20 +/- .13 (SD)
196/429	.08 +/- .05 (SD)

Minimum IR for morphine calibrators, during same period:

414/429	.49
236/429	.61
196/429	.27

RT

0.03 - 0.05 minutes over morphine-d3

Actual R.T. for morphine calibrators

0.00 - 0.02 minutes over morphine-d3.

These data were obtained from a number analytical runs performed in the month of January 1992 and found to remain very close in the above ranges in the intervening months. The ratios are much lower than the corresponding ratios for morphine in all runs where such specimens were detected. The retention times for the GSS peak were always higher than the R.T.'s for morphine. Any combination of lower ratios and/or higher R.T.'s was an indication of the presence of the unknown material.

We also found three types of peaks (see Figure 1):

- 1) Overlapping peaks, where the GSS region was indicated by a shoulder in the peak,

in that either real morphine appeared as a shoulder on the unknown peak, or the unknown material as a shoulder of the morphine peak.

- 2) Partially resolved twin peaks, where the first represented real morphine and the second GSS.
- 3) Single peaks which represented either morphine or GSS.

The morphine peak was characterized by material having the same IR and RT as the morphine used in the calibrators and controls, while GSS had much lower IR. Sometimes an intermediate IR was indicative of a mixture of both materials.

The findings involving GSS were consistent and reproducible, indicating that it is a substance different from that of morphine.

II. Experiments involving variations of the extraction technique

To compare our results with methods used in other laboratories, we repeated the analysis of specimens containing GSS as follows:

- acid hydrolysis with AS and WW procedures;
- beta glucuronidase hydrolysis (bG) with AS and WW procedures.

We used two experimental and two control specimens, at varying concentrations of sample.

Results for the runs carried out with WW columns and both hydrolysis procedures are shown in Figure 2, and in Table 2 below.

SUMMARY OF RESULTS

1. The IR s of the GSS peak, as calculated in the peak obtained by acid hydrolysis appears to match the values seen in section 2, where a similar procedure was used.
2. With experimental samples, acid hydrolysis produced a single peak at a distance of 0.03 minutes from the I.S. This peak showed a lower

concentration of 414 mw fragments, and an extremely reduced level of 236, and 196 fragments.

Enzymatic hydrolysis resolved a single peak into twin peaks, with the first peak having material with the IRs within or close to the morphine range. When both peaks were integrated jointly IRs were obtained which appear to be intermediates between morphine and the unknown substance.

3. With enzymatic hydrolysis, the concentration in the first twin peak calculated manually appeared to be about 50% of the total concentration. The first RT of the twin peaks was roughly equivalent to the RT of morphine.
4. With the control specimen, there was no apparent difference in IRs and RTs between both hydrolytic procedures.

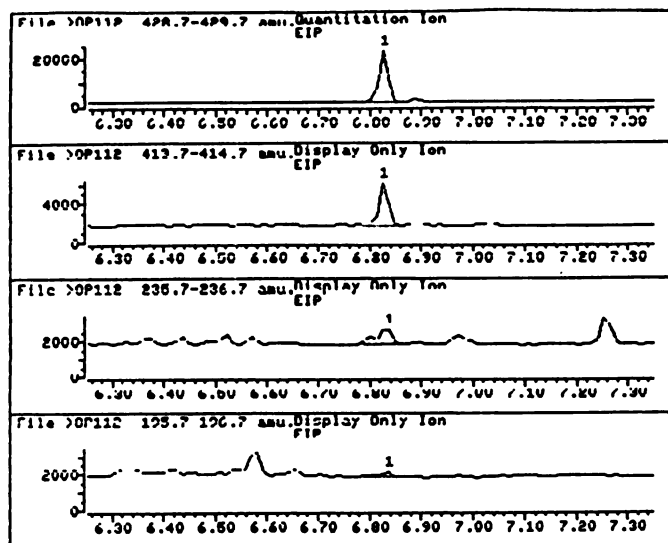
CONCLUSIONS

1. A new peak was observed and confirmed which closely follows morphine, resulting in mixed peaks with lower IRs or not completely resolved peaks, where the first one may represent morphine and the second the unknown entity.

2. The unknown entity (GSS) has lower 414/429 ratios and practically nonexistent 236/429 and 196/429 ratios. Since the 196 ion may represent the (3-ring + nitrogen), $C_{14}H_{13}N$ skeleton of morphine and codeine, it may be inferred that GSS is structurally not closely related to morphine and/or codeine. The presence of 429 and 414 ions may mean that GSS has an exposed $-N-CH_3$ group that loses its methyl ending.
3. While acid hydrolysis produces a single peak with very low IRs, enzymatic hydrolysis generates two peaks, where even the second peak may retain slightly more of the 236 and 196 ions. This may indicate that in this case either: a) acid and enzymatic hydrolysis act on different substrates; or b) enzymatic hydrolysis is more gentle and/or specific.
4. The impact of the techniques used on quantitation has to be better defined with more samples.
5. In control samples where only morphine was present the type of procedure had no substantive impact on the results obtained.
6. More research is being carried out to gain further information on the identity of this new entity, and to make it possible to accurately confirm and report morphine.

Table 2. RT and IR's of Two Experimental and One Control Samples Confirmed Using Both bG and Acid Hydrolysis.						
Calibrator IR Ranges	1) 414/429		2) 236/429		3) 196/429	
Acid hydrolysis (AC)	.37 - .56		.94 - 1.41		.53 - .80	
Glucuronidase (BG)	.40 - .60		1.15 - 1.73		.66 - .99	
	RT Sample/IS	IR 1	IR 2	IR 3	Conc. ng/ml	Peak Shape
6-0416-Exp						
AC	6.04/6.01	.22#	.12#	.05#	a*	Single
BG	6.02/6.02	.37#	.73#	.37#	2407	1 + 2**
	6.02/6.02	.45	1.04#	.50#	1466	1**
6-0504-Exp						
AC	6.03/6.00	.31#	.41#	.16#	a*	Single
BG	6.03/6.02	.40#	.82#	.39#	401	1 + 2**
	6.02/6.02	.49	1.16	.52#	236	1**
13-0418-Control						
AC	6.02/6.00	.56	1.14	.53	747	Single
BG	6.01/6.01	.51	1.18	.55#	548	Single
<p># - Out of calibrator IR range.</p> <p>a* - Concentration of analyte obtained with acid hydrolysis was normally higher than the one obtained enzymatically. This relationship has to be further elucidated.</p> <p>** - 1 and 2 denote peak 1 and 2 of the twin peak produced by bG hydrolysis. (1 + 2) denotes that the peaks were integrated together by the instrument. (1) denotes manual integration of the first peak.</p>						

Figure 1-1. GSS peak only.



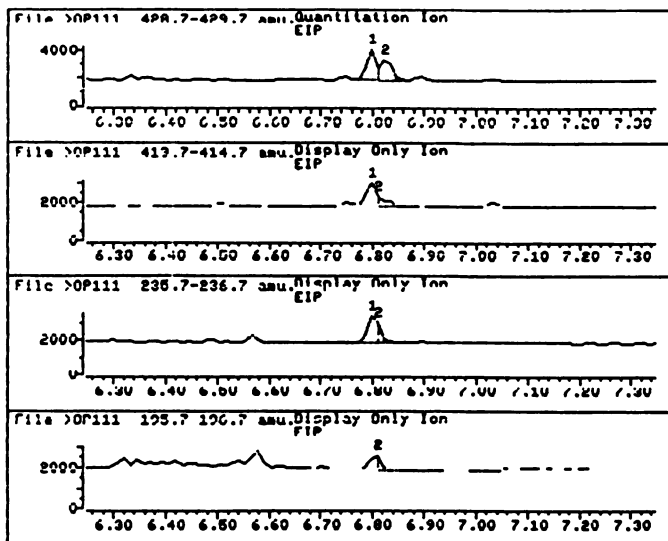
Data File: >OP112::A2
 Name: 22-0535
 Misc: F328602 X10
 Quant Time: 920125 01:58
 Injected at: 920125 01:45
 Last Qcal Time: <none>

Quant Output File: ^OP112::QT
 Instrument ID: MSD #2
 Quant ID File: IOPS::SC
 Last Calibration: 920124 17:03
 BTL#39

Compound No : 4
 Compound Name: Morphine
 Quant Ion : 429.0

Hit R.T.		Conc		Areas and Ratios			
#	Scan#	Area	Units	q	429.0	414.0	236.0
1	6.83	24234.47	NG/ML	100	26710	5503	1518
	358	26710			1.000	.206	.057

Figure 1-1. GSS peak separated into twin peaks. Peak 1 as intermediate, peak 2 GSS.



Data File: >OP111::A2
 Name: 22-0522
 Misc: F328589
 Quant Time: 920125 01:42
 Injected at: 920125 01:29
 Last Qcal Time: <none>

Quant Output File: ^OP111::QT
 Instrument ID: MSD #2
 Quant ID File: IOPS::SC
 Last Calibration: 920124 17:03
 BTL#38

Compound No : 4
 Compound Name: Morphine
 Quant Ion : 429.0

Hit R.T.		Conc		Areas and Ratios			
#	Scan#	Area	Units	q	429.0	414.0	236.0
1	6.80	125.72	NG/ML	100	2741	1490	2135
	352	2741			1.000	.544	.779
2	6.82	83.49	NG/ML	100	1979	433	257
	354	1979			1.000	.219	.130

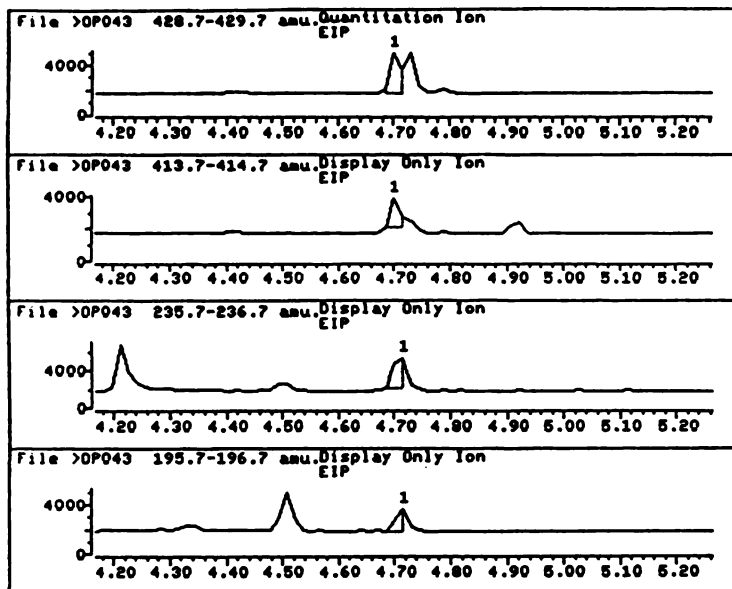


Figure 1-3. GSS peak separated into twin peaks. Peak 1 is morphine.

Data File: >OP043::A2 Quant Output File: ^OP043::QT
 Name: 4-0478 Instrument ID: MSD #2
 Misc: F370714 X10 BTL#10
 Quant Time: 920608 11:48 Quant ID File: IOPS::SC
 Injected at: 920608 11:34 Last Calibration: 920608 10:45
 Last Qcal Time: <none>

Compound No : 4
 Compound Name: Morphine
 Quant Ion : 429.0

Hit R.T.					Areas and Ratios			
#	Scan#	Area	Units	q	429.0	414.0	236.0	196.0
1	4.70	762.40	NG/ML	100	4518M	2114m	5424m	2268m
	82	4518M			1.000	.468	1.201	.502

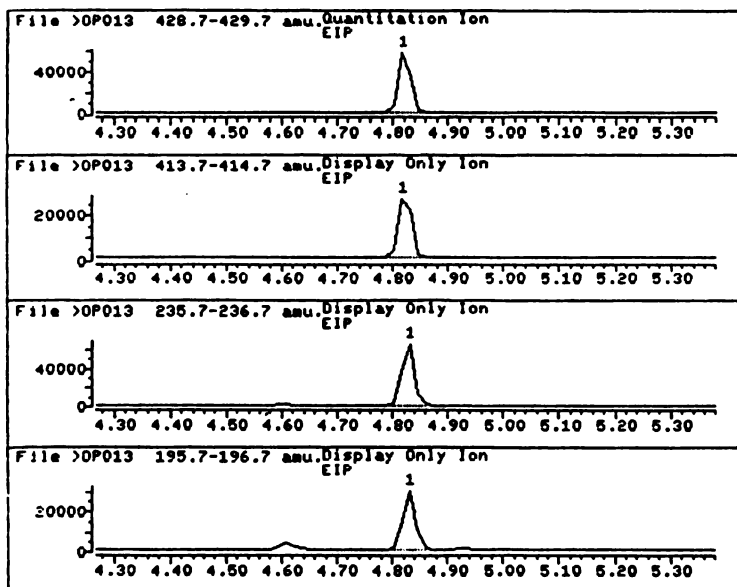
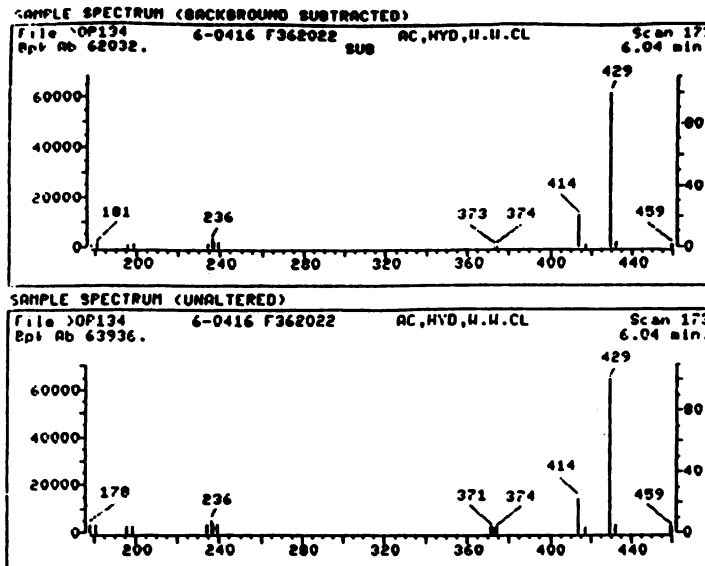


Figure 1-4. Control morphine.

Data File: >OP013::A2 Quant Output File: ^OP013::D0
 Name: 15-0494 Instrument ID: MSD #2
 Misc: F364970 X10 BTL# 9
 Quant Time: 920523 12:22 Quant ID File: IOPS::SC
 Injected at: 920523 11:27 Last Calibration: 920523 12:06
 Last Qcal Time: <none>

Compound No : 4
 Compound Name: Morphine
 Quant Ion : 429.0

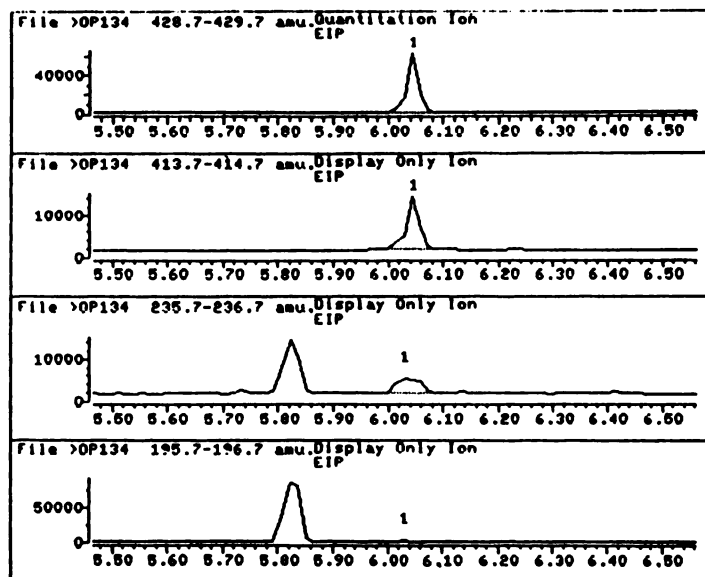
Hit R.T.					Areas and Ratios			
#	Scan#	Area	Units	q	429.0	414.0	236.0	196.0
1	4.82	9598.87	NG/ML	100	90547	45357	102328	44866
	91	90547			1.000	.501	1.130	.495



Data File: >OP134::D0 Quant Output File: ^OP134::QT
 Name: 6-0416 F362022 Instrument ID: MSD #2
 Misc: AC,HYD,W.W.CL BTL#44
 Quant Time: 920611 12:19 Quant ID File: IOPS::SC
 Injected at: 920611 00:59 Last Calibration: 920611 11:59
 Last Qcal Time: <none>

Compound No : 4
 Compound Name : Morphine
 Scan Number : 173
 Retention Time: 6.04 min.
 Quant Ion : 429.0
 Area : 92910
 Concentration : 114047.4 NG/ML
 q-value : 100

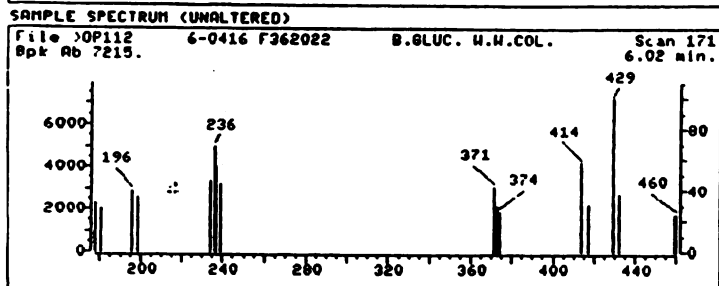
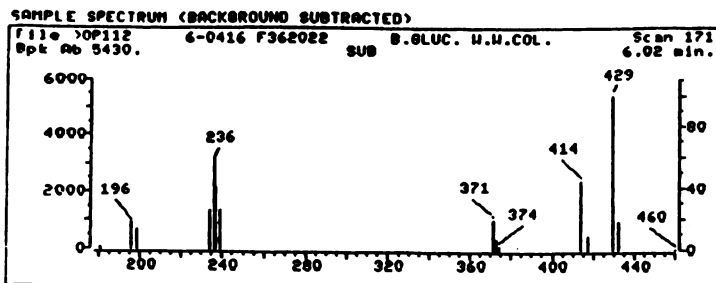
Figure 2-1. Acid hydrolysis, WW columns. Top: ions; bottom: peak.



Data File: >OP134::D0 Quant Output File: ^OP134::QT
 Name: 6-0416 F362022 Instrument ID: MSD #2
 Misc: AC,HYD,W.W.CL BTL#44
 Quant Time: 920611 12:19 Quant ID File: IOPS::SC
 Injected at: 920611 00:59 Last Calibration: 920611 11:59
 Last Qcal Time: <none>

Compound No : 4
 Compound Name : Morphine
 Quant Ion : 429.0

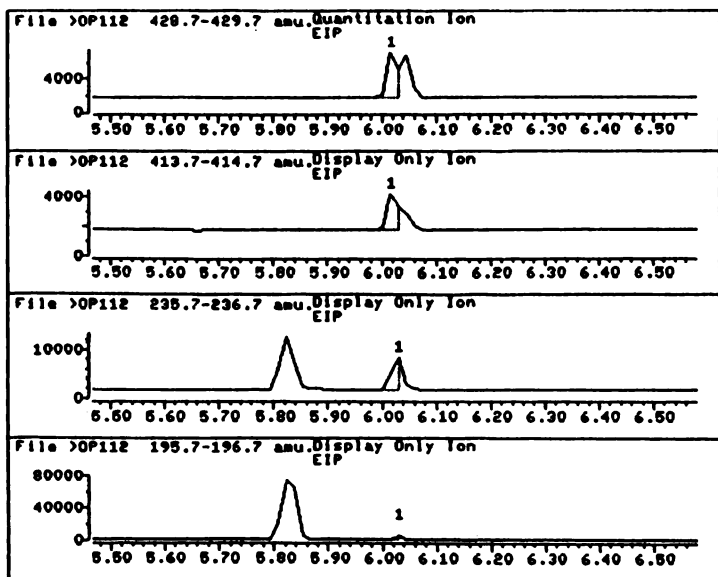
Hit R.T. Conc				Areas and Ratios			
#	Scan#	Area	Units	q	429.0	414.0	236.0
1	6.04	114047.4	NG/ML	100	92910	20915	11691
	173	92910			1.000	.225	.126



Data File: >OP112::D0 Quant Output File: ^OP112::QT
 Name: 6-0416 F362022 Instrument ID: MSD #2
 Misc: B.GLUC. W.W.COL. BTL#22
 Quant Time: 920611 08:55 Quant ID File: IOPS::SC
 Injected at: 920610 18:59 Last Calibration: 920611 07:50
 Last Qcal Time: <none>

Compound No : 4
 Compound Name: Morphine
 Scan Number : 171
 Retention Time: 6.02 min.
 Quant Ion : 429.0
 Area : 8330M
 Concentration : 1466.18 NG/ML
 q-value : 100

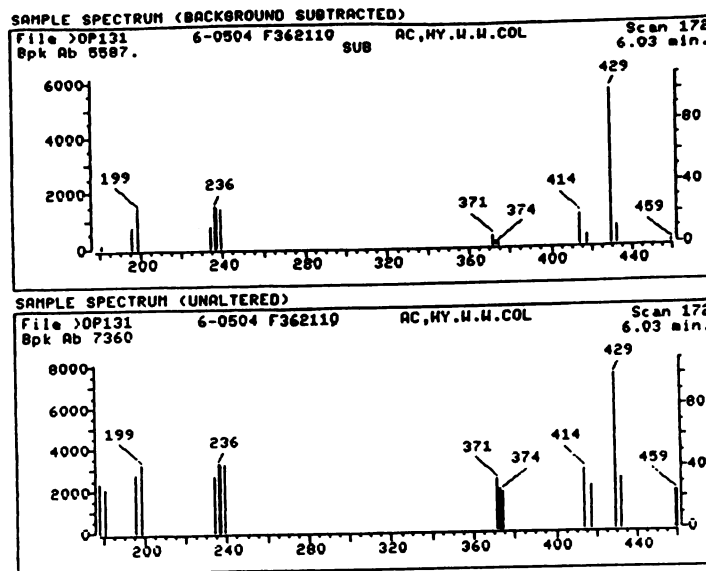
Figure 2-1. Enzymatic hydrolysis, WW columns. Top: ions; bottom: peaks. Peak 1 is morphine, peak 2 is GSS.



Data File: >OP112::D0 Quant Output File: ^OP112::QT
 Name: 6-0416 F362022 Instrument ID: MSD #2
 Misc: B.GLUC. W.W.COL. BTL#22
 Quant Time: 920611 08:55 Quant ID File: IOPS::SC
 Injected at: 920610 18:59 Last Calibration: 920611 07:50
 Last Qcal Time: <none>

Compound No : 4
 Compound Name: Morphine
 Quant Ion : 429.0

Hit	R.T.	Conc	Areas and Ratios			
#	Scan#	Area	Units	q	429.0	414.0
1	6.02	1466.18	NG/ML	100	8330M	3759m
	1/1	8330M			1.000	.451

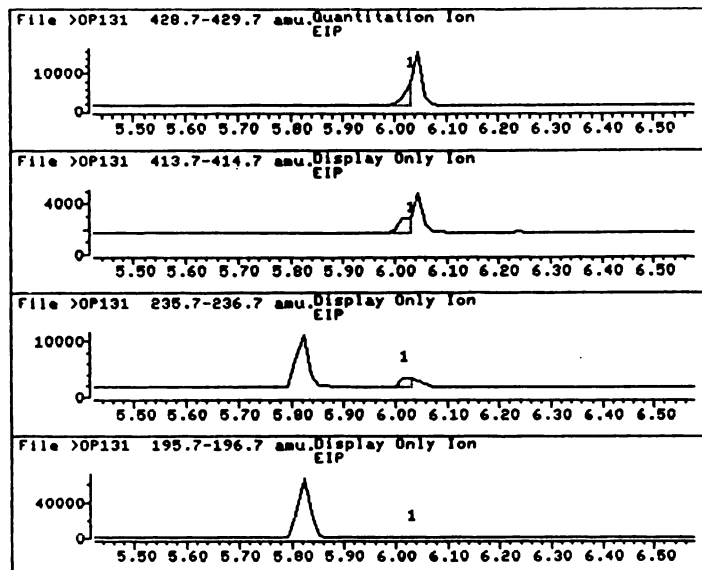


Date File: >OP131::D0
Name: 6-0504 F362110
Misc: AC, HY.W.W.COL
Quant Time: 920611 12:09
Injected at: 920610 23:59
Last Qcal Time: <none>

Quant Output File: ^OP131::QT
Instrument ID: MSD #2
BTLE#41
Quant ID File: IOPS::SC
Last Calibration: 920611 11:59

Compound No : 4
Compound Name : Morphine
Scan Number : 172
Retention Time: 6.03 min.
Quant Ion : 429.0
Area : 6851m
Concentration : 378.47 NG/ML
q-value : 100

Figure 2-2. Acid hydrolysis,
WW columns. Top: ions;
bottom: peak.

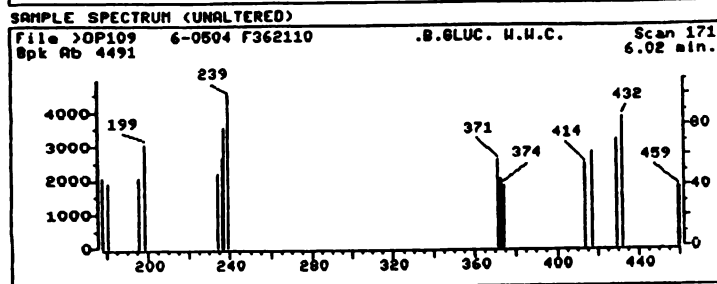
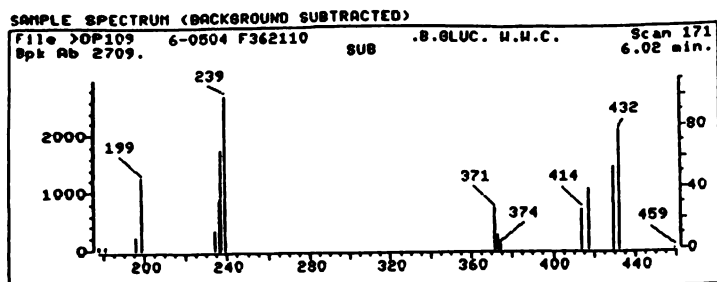


Date File: >OP131::D0
Name: 6-0504 F362110
Misc: AC, HY.W.W.COL
Quant Time: 920611 12:09
Injected at: 920610 23:59
Last Qcal Time: <none>

Quant Output File: ^OP131::QT
Instrument ID: MSD #2
BTLE#41
Quant ID File: IOPS::SC
Last Calibration: 920611 11:59

Compound No : 4
Compound Name: Morphine
Quant Ion : 429.0

Hit R.T.				Areas and Ratios			
#	Scan#	Area	Units	q	429.0	414.0	236.0
1	6.03	378.47 NG/ML	100	6851m	2145M	2826m	1157m
	172	6851m		1.000	.313	.412	.169

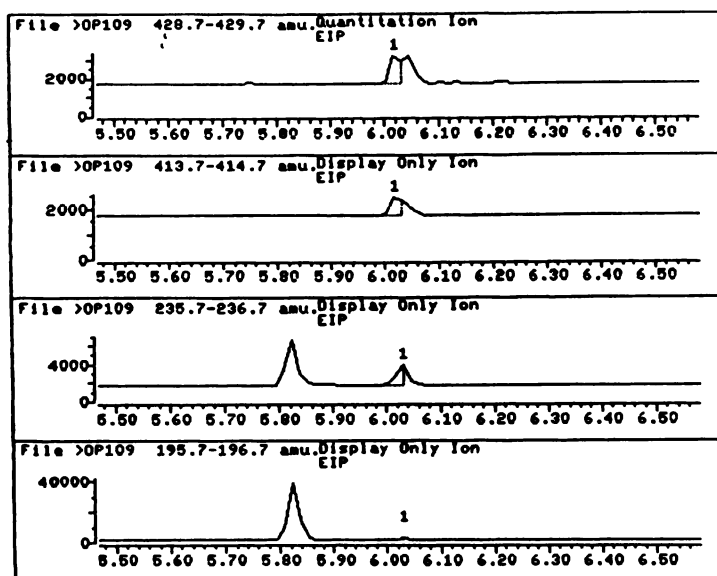


Data File: >OP109::D0
Name: 6-0504 F362110
Misc: .B.GLUC. W.W.C.
Quant Time: 920611 08:32
Injected at: 920610 17:59
Last Qcal Time: <none>

Quant Output File: ^OP109::QT
Instrument ID: MSD #2 BTL#19
Quant ID File: IOPS::SC
Last Calibration: 920611 07:50

Compound No : 4
Compound Name : Morphine
Scan Number : 171
Retention Time: 6.02 min.
Quant Ion : 429.0
Area : 2386M
Concentration : 236.23 NG/ML
q-value : 100

Figure 2-2. Enzymatic hydrolysis, WW columns. Top: ions; bottom: peaks. Peak 1 is morphine, peak 2 is GSS.

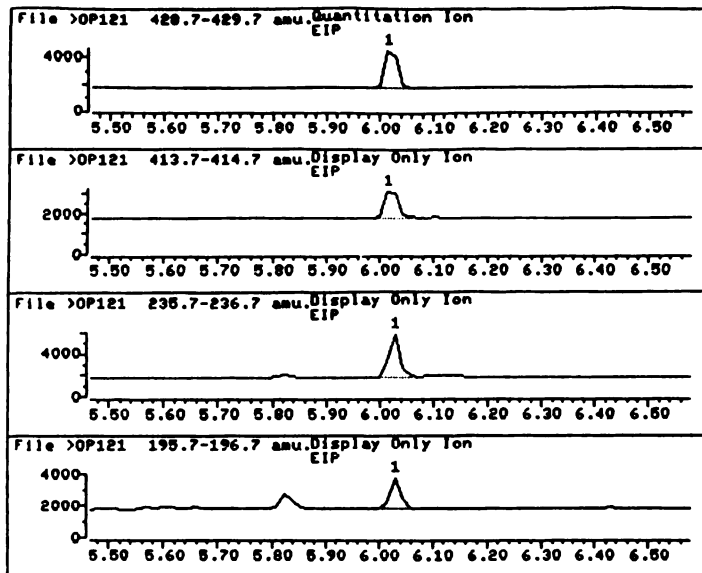


Data File: >OP109::D0
Name: 6-0504 F362110
Misc: .B.GLUC. W.W.C.
Quant Time: 920611 08:32
Injected at: 920610 17:59
Last Qcal Time: <none>

Quant Output File: ^OP109::QT
Instrument ID: MSD #2 BTL#19
Quant ID File: IOPS::SC
Last Calibration: 920611 07:50

Compound No : 4
Compound Name: Morphine
Quant Ion : 429.0

Hit R.T.		Conc		Areas and Ratios			
#	Scan#	Area	Units	q	429.0	414.0	236.0
1	6.02	236.23	NG/ML	100	2386M	1181m	2780m
	171	2386M			1.000	.495	1.165

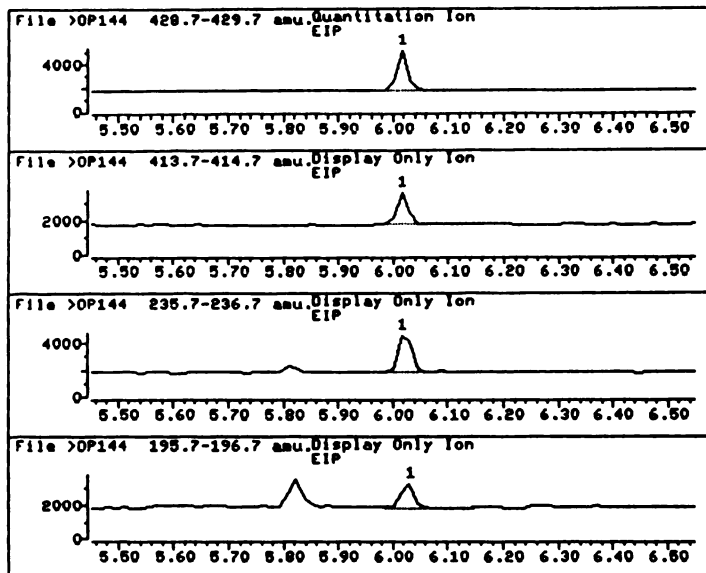


Data File: >OP121::D0 Quant Output File: ^OP121::QT
 Name: 13-0418 F364074 Instrument ID: MSD #2 BTL#31
 Misc: B.GLUC.W.W.COL.X2.0
 Quant Time: 920611 09:26 Quant ID File: IOPS::SC
 Injected at: 920610 21:29 Last Calibration: 920611 07:50
 Last Qcal Time: <none>

Compound No : 4
 Compound Name: Morphine
 Quant Ion : 429.0

					----- Areas and Ratios -----			
Hit #	R.T.	Conc	Area	Units	q	429.0	414.0	236.0
1	6.01	548.51	NG/ML	100	4771	2452	5641	2661
	171	4771			1.000	.514	1.182	.558

Figure 2-3. Control morphine.
 Top: enzymatic hydrolysis;
 bottom: acid hydrolysis.



Data File: >OP144::D0 Quant Output File: ^OP144::QT
 Name: 13-0418 F364074 Instrument ID: MSD #2 BTL#54
 Misc: AC.HYD.W.W.COL X2.0
 Quant Time: 920611 12:39 Quant ID File: IOPS::SC
 Injected at: 920611 03:28 Last Calibration: 920611 11:59
 Last Qcal Time: <none>

Compound No : 4
 Compound Name: Morphine
 Quant Ion : 429.0

					----- Areas and Ratios -----			
Hit #	R.T.	Conc	Area	Units	q	429.0	414.0	236.0
1	6.02	747.25	NG/ML	100	4696	2671	5365	2505
	171	4696			1.000	.569	1.142	.533

Atmospheric Sampling of Cocaine Emission During Large-Scale Drug Destruction Burns

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Sacramento, CA

In October 1989, cocaine was declared a hazardous waste in California. With this designation, all previously routine incineration of cocaine contraband was halted. This paper describes a large-scale test burn and subsequent air and residue analysis for cocaine.

The test burn was conducted at the U.S. Mint in San Francisco employing a scrubberless, one-stage incinerator operating at 1900°F. Approximately 1,200 pounds of confiscated cocaine (purity 90+%) was burned over a 6-hour period. Particulate sampling was performed using a modified CAL EPA method five train, which samples stack gasses isokinetically through a glass filter and wet impingers. These modifications included using a 0.1 N HCl solution instead of water in the impingers to improve cocaine stability, and removal of the solid-phase column between the filter and the impingers. Ash samples were also analyzed for cocaine. Because the behavior of cocaine using this sampling method was unknown, a validation study was conducted in the laboratory using a low-temperature controlled burn and identical sampling train.

All analyses of the probe rinse, line rinse, filter digest, and impinger solutions (all 0.1 N HCl) were identical. Samples and blanks were initially screened by radioimmunoassay using DPC Coat-a-Count for cocaine metabolite (Diagnostic Products Corporation, Los Angeles, CA). Subsequent extraction of remaining samples (each approximately 50 milliliters) was performed with 2 x 100 ml ethyl acetate after pH

adjustment by saturation with sodium bicarbonate (pH 8.5). Dried extracts were analyzed by GC/MS and GC/MS/MS to detect cocaine (Davis 1991).

Results of the validation study indicate that most intact cocaine ends up on the glass filter, with decreasing amounts present on probe, filter line, and impinger solutions respectively. Analysis of the large-scale burnt raining components indicate absence of cocaine at a detection limit of 500 parts per trillion (ppt). Additional analysis of the ash samples was performed by the California Department of Toxic Substances Control Laboratory with no cocaine detected at a 10 microgram per gram cutoff. A subsequent test burn at another facility was conducted and the training components analyzed by an independent laboratory. The results for cocaine in the emission gas were negative, but ash samples contained approximately 200 - 300 ppb cocaine.

By these results, a conclusion can be reached that large-scale incineration is an efficient and thorough method of cocaine destruction with virtually no cocaine being introduced into the atmosphere.

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Workplace Testing of Nordiazepam and Oxazepam: A Potential Pitfall in GC/MS Confirmation

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New Hyde Park, NY

Benzodiazepine analysis is a growing area in workplace testing. The NRC, CAP, and governmental agencies such as those in the states of New York and Florida require drug-testing laboratories to test and quantify benzodiazepines. Additionally, there are indications that NIDA is considering expansion of the current five drug categories to perhaps include benzodiazepines. However, in the case of benzodiazepines different GC/MS methodologies may yield quantitative levels of oxazepam and/or nordiazepam that are vastly different from each other. This has the potential to create gross inconsistencies in results and hence to pose significant problems in result interpretation.

We analyzed for nordiazepam and oxazepam by two different GC/MS methods. The initial method was a modified version of the procedure of Maurer and Pfleger (1981) and was based on acid hydrolysis to yield 2-amino-5-chloro-benzophenone (ACB). Internal standards, oxazepam-d5, and nordiazepam-d5, (Radian Corporation, Austin, TX), were added to the urine specimens. Next, concentrated hydrochloric acid was used to hydrolyze at 65°C for 30 minutes. The reaction was stopped by the addition of concentrated ammonium hydroxide. The hydrolysate was applied to a Jetube (Har-Len Medical, Inc., Gibsonia, PA). ACB and ACB-d5 were eluted with dichloromethane: isopropanol (9:1). The eluate was evaporated at 37°C; the residue was reconstituted with methanol; and 1 - 2 mL injected onto a Hewlett-Packard 5970 GC/MS system. GC/MS analysis was in the SIM mode and m/z ions 230, 231, 232, and 233 were monitored for ACB and m/z ions 234 and 236 were monitored for ACB-d5. The

observed levels of ACB derived from nordiazepam are approximately half of those formed from oxazepam. Quantitation with ACB utilizing oxazepam as a calibrator yielded levels of ACB derived from nordiazepam that are listed in Table 1. Whereas, quantitation with ACB using nordiazepam as a calibrator demonstrated the levels of ACB derived from oxazepam that are listed in Table 2.

The second method used was a modified procedure of Mule and Casella (1989). This method employed enzyme hydrolysis and trimethylsilyl derivatization with BSTFA and 1% TMCS. Nordiazepam-d5 and oxazepam-d5 were added to the urine specimens. Next, glucuronidase (Sigma Chemical Company, St. Louis, MO) and a 2.0 N acetate buffer (pH 4.8) were added and the mixture allowed to incubate at 55°C for at least 2 hours (Marion Laboratories, Inc., and Hewlett-Packard Company 1987). The reaction was stopped by allowing the tubes to cool and adding a KCl/NaOH buffer (pH about 12) to each tube. Dichloromethane was added; the contents of each tube were mixed on a rotating mixer for 5 minutes. The tubes were centrifuged for 5 minutes and the top aqueous layer discarded. Diluted KCl/NaOH buffer was added and the contents of each tube were mixed for 5 minutes. Tubes were then centrifuged for 5 minutes and the aqueous layer was discarded. The dichloromethane phase was evaporated at 37°C. The residue was dissolved in BSTFA with 1% TMCS and the reaction allowed to proceed for 15 minutes at 65°C. 1 - 2 mL was injected onto the GC/MS system. Analysis was done in the SIM mode. The m/z ions for TMS-nordiazepam were 327, 341, 342, and 343; the m/z ions for TMS-

nordiazepam-d5 were 332, 345, and 347. The m/z ions for TMS-oxazepam were 415, 429, 430, and 431; the m/z ions for TMS-oxazepam-d5 were 420, 433, and 435. Because this method yields two different derivatives for oxazepam and nordiazepam, the problem that arises with ACB formation does not occur. Each of the two products has its own calibrator which results in consistently accurate and reliable quantitation as shown in Tables 3 and 4.

In summary, GC/MS confirmation of workplace testing of nordiazepam and oxazepam was found to provide significantly different quantitative results depending on the method used. Our study indicates that calibrators should consist of both oxazepam and nordiazepam, and that enzyme hydrolysis with trimethylsilyl derivatization is a preferred analytical methodology.

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- Mule, S. and Casella, G. Quantitation and confirmation of the diazolo- and triazolobenzodiazepines in human urine by gas chromatography/mass spectrometry, *Journal of Analytical Toxicology* (1989) 13:179-184.

Table 1. Recovery of ACB Produced by Acid Hydrolysis of Nordiazepam Spiked Urine Samples Utilizing Oxazepam Calibrators.	
Spiked Nordiazepam Levels (ng/mL)	ACB Results (ng/mL)
150	88
300	138
600	270

Table 2. Recovery of ACB Produced by Acid Hydrolysis of Oxazepam Spiked Urine Samples Utilizing Nordiazepam Calibrators.	
Spiked Oxazepam Levels (ng/mL)	ACB Results (ng/mL)
300	648
1000	2067
1500	2718

Table 3. Recovery of Nordiazepam After Enzyme Hydrolysis and Trimethylsilyl Derivatization of Spiked Urine Samples Utilizing Nordiazepam Calibrators.	
Spiked Nordiazepam Levels (ng/mL)	Nordiazepam Results (ng/mL)
150	148
225	238
375	416
600	600
1500	1360

Table 4. Recovery of Oxazepam After Enzyme Hydrolysis and Trimethylsilyl Derivatization of Spiked Urine Samples Utilizing Oxazepam Calibrators.	
Spiked Oxazepam Levels (ng/mL)	Oxazepam Results (ng/mL)
500	508
750	805
1000	1013
1500	1401

Dynamics of the Blood-Alcohol Curve of Social Drinkers

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Most drinking experiments have studied the consumption of alcohol as a bolus quantity and the resulting blood-alcohol curve (Jones *et al.* 1991). A few works have considered the consumption of alcohol at regular intervals over a longer period of time which show a blood-alcohol curve which differs from bolus ingestion (Shajani and Dinn 1985; Jones and Neri 1991).

The research presented in this work differs in that it studies the blood-alcohol (BA) curve for social drinkers (*i.e.*, those people who consume alcohol at their own rate). The drinking was conducted over a period of time consistent with persons who may normally be encountered in driving under the influence situations.

In most states, it is necessary in legal proceedings to establish a subject's BA value at the time of driving or accident from a test (breath or blood) taken an hour or longer after the time of interest.

This study was designed to look at several factors which address this issue.

- 1) The time to maximum BA after the last drink.
- 2) The effect of "one last drink" shortly before drinking ceases.
- 3) The dynamics of the BA curve and how this relates to retrograde extrapolation.

MATERIALS AND METHODS

Thirty-eight subjects drank their choice of various alcoholic beverages and consumed food (one experiment was conducted in which the subjects did not eat) in a

semicontrolled social-scientific setting. At a predetermined time, all alcohol consumption was terminated. The drinking session lasted from 2 - 3 hours and the consumption was not predetermined, but rather the subjects were allowed to drink as they wished.

Monitors at the experiments accurately recorded the start and finishing time of each drink, the times of blood samples and breath tests, and the amount and type of alcohol ingested.

RESULTS

The normal social drinking pattern (Storm and Cutter 1981) results in a BA curve which in many cases differs from the typical curves which have been published relating to bolus or constant rate consumption. Our observations in this research and other work we have conducted (not detailed in this report) show that individuals drinking in a social setting consume larger quantities at the first of a drinking period rather than later in the drinking period. Social drinkers also usually consume some type of food along with the alcohol. The BA curve for most of these individuals (Charts 1, 2, 3, and 4) is much flatter near the peak and results in "plateauing," sometimes for long periods of time (1 - 3 hours). It was also observed that "one last drink" even when consumed rapidly at the end of the drinking period had little effect on the peak BA level (less than .01%), but rather may prolong the plateau.

DISCUSSION

The setting or drinking pattern of an individual is important when back extrapolation is conducted. When called upon to provide expert testimony the range given for the BA value will have greater certainty if the much flatter BA curve is considered. It must be

recognized that a subject's BA value an hour or two prior to a test is not always significantly different. This may also assist in the "hip flask" defense situations which suggest greatly rising or falling BA curves. It is important to know the conditions under which the subject consumed the alcohol. A social setting, drinking time, etc. will effect the dynamics of the alcohol curve.

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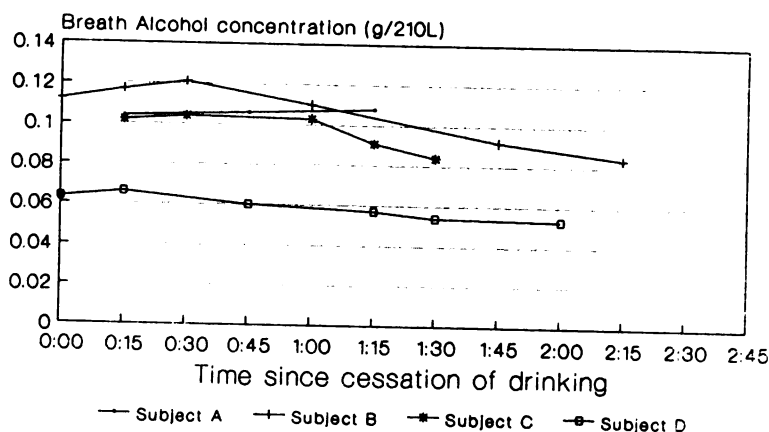
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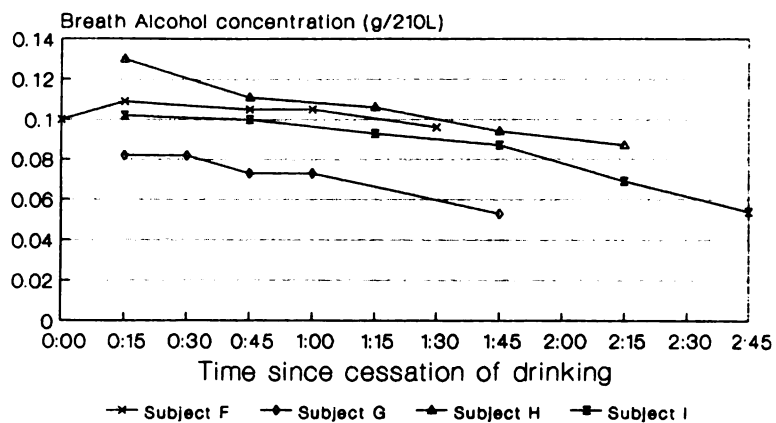
ALCOHOL DISSIPATION AFTER SOCIAL DRINKING



FULL STOMACH

Chart 1.

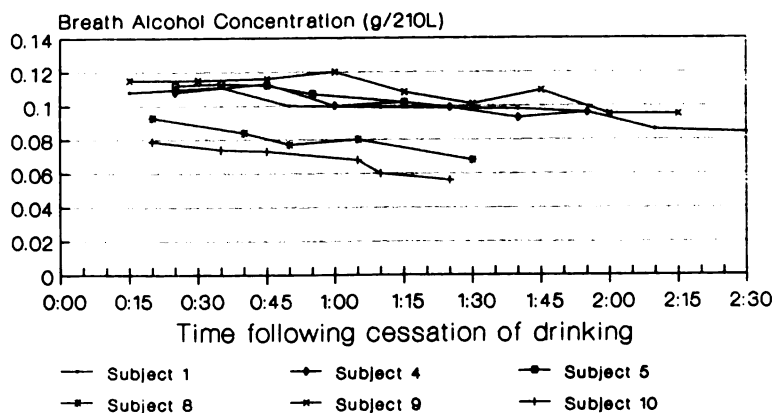
ALCOHOL DISSIPATION AFTER SOCIAL DRINKING



EMPTY STOMACH

Chart 2.

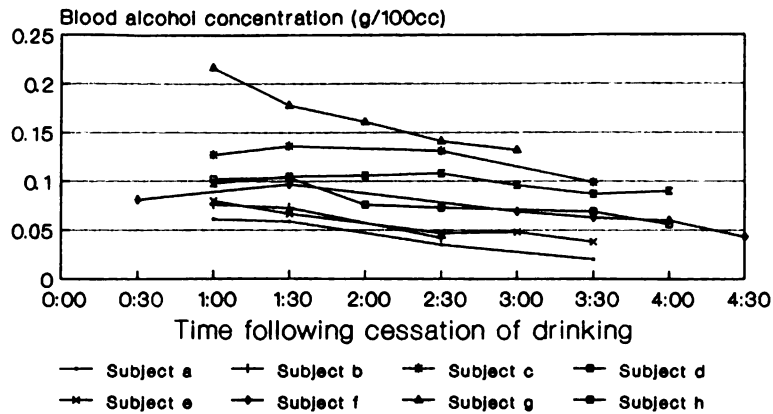
ALCOHOL DISSIPATION AFTER SOCIAL DRINKING



FOOD AVAILABLE

Chart 3.

ALCOHOL DISSIPATION AFTER SOCIAL DRINKING



FOOD AVAILABLE

Chart 4.

A Multiple Drug Intoxication Involving Cyclobenzaprine and Ibuprofen

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A 19-year-old black male was presented to the emergency room demonstrating fatigue and lethargy. There were superficial cuts on the anterior wrists. Gastric lavage yielded tablet fragments. Available medications include cyclobenzaprine, ibuprofen, and an over-the-counter decongestant. Seizures and a metabolic acidosis developed and the patient died 3 hours post-admission. An autopsy failed to provide an anatomic cause of death. Blood, vitreous fluid, stomach contents, and tissues were sent to the toxicology laboratory for analysis.

MATERIALS AND METHODS

Comprehensive testing for alcohol and drugs was performed on the blood specimens received. Methanol, ethanol, acetone, C₃, and C₄ volatiles were tested by head space gas chromatography. A drug of abuse screen utilized radioimmunoassay (Roche Diagnostics, Nutley, NJ). Basic drugs were identified by gas chromatography-nitrogen-phosphorus detection (Watts and Simonick 1986). Salicylate, acetaminophen, and phenytoin were tested by fluorescence polarization immunoassay (Abbott Diagnostics, Abbott Park, IL). All positive screening results were confirmed by full scan electron impact gas chromatography-mass spectrometry (GC/MS).

All identified drugs were quantitated in appropriate specimens using in-house modifications of previously published methods by either gas chromatography or GC/MS (Watts and Simonick 1986; Baselt 1980; Von Minden and D'Amato 1977).

RESULTS

The analytical results obtained in this case are given in Table 1.

DISCUSSION

The collection of the toxicology results was straightforward. All drugs except ibuprofen were identified by the routine screening process. A special assay for ibuprofen involving an acid extraction, methylation, and a clean-up extraction was developed. Phenytoin and phenylpropanolamine were quantitated as their methylated and chlorodifluoroacetylated derivatives, respectively.

The interpretation of the toxicology results in this case is complex. The lidocaine and phenytoin detected were administered therapeutically. The phenylpropanolamine and chlorpheniramine concentrations are above the reported antemortem therapeutic ranges, but well below concentrations reported in documented overdoses. The cyclobenzaprine concentration is about an order of magnitude above the reported antemortem therapeutic range, but is less than the concentration observed in the two cyclobenzaprine fatalities reported (Beck and Lamoreaux 1979). The ibuprofen concentration is above that found in one reported fatality (Steinmetz *et al.* 1987), but much higher blood concentrations patients who survived. In this case, the clinical history is quite important in sorting out these inconsistencies. One of the symptoms

displayed by the patient was metabolic acidosis. One of the toxic effects produced by an overdose of ibuprofen is metabolic acidosis (Hall, *et al.* 1986). One other symptom demonstrated by the patient was seizures, a toxic effect of cyclobenzaprine intoxication.

CONCLUSION

By combining the clinical history with the toxicology findings, the medical examiner ruled that the cause of death was multiple drug intoxication. The manner of death was suicide.

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Table 1. Analytical Results.

Specimen	Concentration					
	CBP	IBU	CHL	PPA	LID	DPH
Blood (mg/L)	0.3	130	0.2	2.5	6.6	19
Brain (mg/kg)	6.5	1600	—	—	—	—
Kidney (mg/kg)	2.4	270	—	—	—	—
Liver (mg/kg)	7.1	880	—	—	—	—
Stomach Contents (mg)	1.4	115	—	—	—	—
CBP = Cyclobenzaprine IBU = Ibuprofen CHL = Chlorpheniramine PPA = Phenylpropanolamine LID = Lidocaine DPH = Penytain — = Analysis Not Performed						

Trends in Fatal Poisoning and Forensic Chemical Examinations of Paraquat Poisoning in Japan

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It may be a distinctive feature in Japan that one third of the annual fatal poisoning cases have arisen by pesticide, especially by paraquat and diquat (more than 1,200 cases in 1986).

For screening of paraquat and diquat, we have developed a excellent extraction method from various specimens using a disposable cartridge of ODS. Confirmation and identification of the herbicides have been performed by colorimetry, GC/MS, and GC/FTIR.

MATERIALS AND METHODS

Paraquat dichloride and diquat dibromide (ICI Japan, Tokyo) were dried at 110°C for 2 hours prior to use. Sep-Pak C18 cartridge was purchased from Waters, Division of Millipore (Milford, MA).

GC/MS analyses were performed using a Perkin-Elmer 8420 gas chromatograph on a DB-17 fused silica capillary column (J & W Scientific, Folsom, CA) equipped with ion trap detector (ITD). Oven temperature was isothermal at 200°C, or temperature-programmed from 100°C to 200°C (40°C/minute, split mode). GC/FTIR were carried out on a Hewlett-Packard 5890 gas chromatograph equipped with HP 5965A IRD. Column oven was 200°C. The others were same as the GC/MS conditions (Tsunoda and Ohtsuru 1989).

The extraction of samples have been carried out as follows (Tsunoda 1983). A sample solution adjusted

to pH 10 with sodium carbonate, is passed through a preconditioned Sep-Pak C18 cartridge. The cartridge is washed with water, methanol, and water. Paraquat and diquat are eluted with 0.1 N hydrogen chloride from the cartridge. The collected eluate is used for assay.

Paraquat and diquat are determined colorimetrically after reduction of an eluate with alkaline sodium dithionite.

After reduction of an aliquot of the eluate with a mixture of sodium borohydride-nickel (II) chloride (Kawase *et al.* 1984), the reduction products were submitted for confirmation of paraquat and diquat by GC/MS and GC/FTIR (Tsunoda and Ohtsuru 1989).

RESULTS

Paraquat and diquat were retained on the Sep-Pak C18 cartridge around pH 10 and recovered quantitatively from the cartridge by eluting with 0.1 N hydrogen chloride (Figure 1). The extraction time is approximately 10 minutes for a 10 ml urine sample. Washing the cartridge with methanol after passing though a sample is very effective to remove impurities of various samples. A DB-17 column was suitable for the simultaneous detection and confirmation of paraquat and diquat as their perhydrogenated products. Further enhancement of specificity was achieved by MS at the molecular ions of the perhydrogenated products (m/z 196 and 194) and their base peak ions (m/z 96 and 83) as shown in Figure 2, and also done by IRD.

Using this extraction method and colorimetry, we have determined 5 ug of paraquat in 2,700 ml of the urine sample, and identified by GC/MS as the perhydrogenated product.

CONCLUSIONS

This extraction method combined with colorimetry and/or GC/MS is recommended to perform the chemical examination of paraquat and diquat in the forensic laboratories and emergency units.

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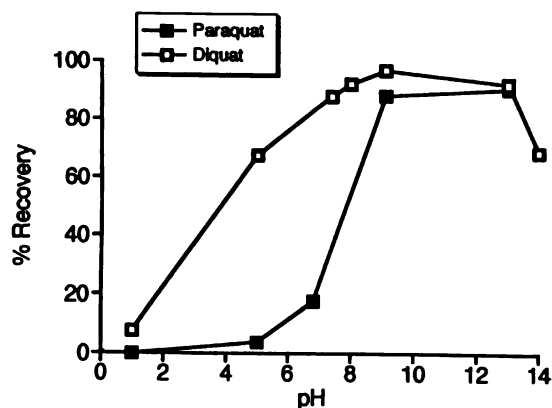


Figure 1. Effects of pH on Retention of Paraquat and Diquat on Sep-Pak C18 Cartridge.

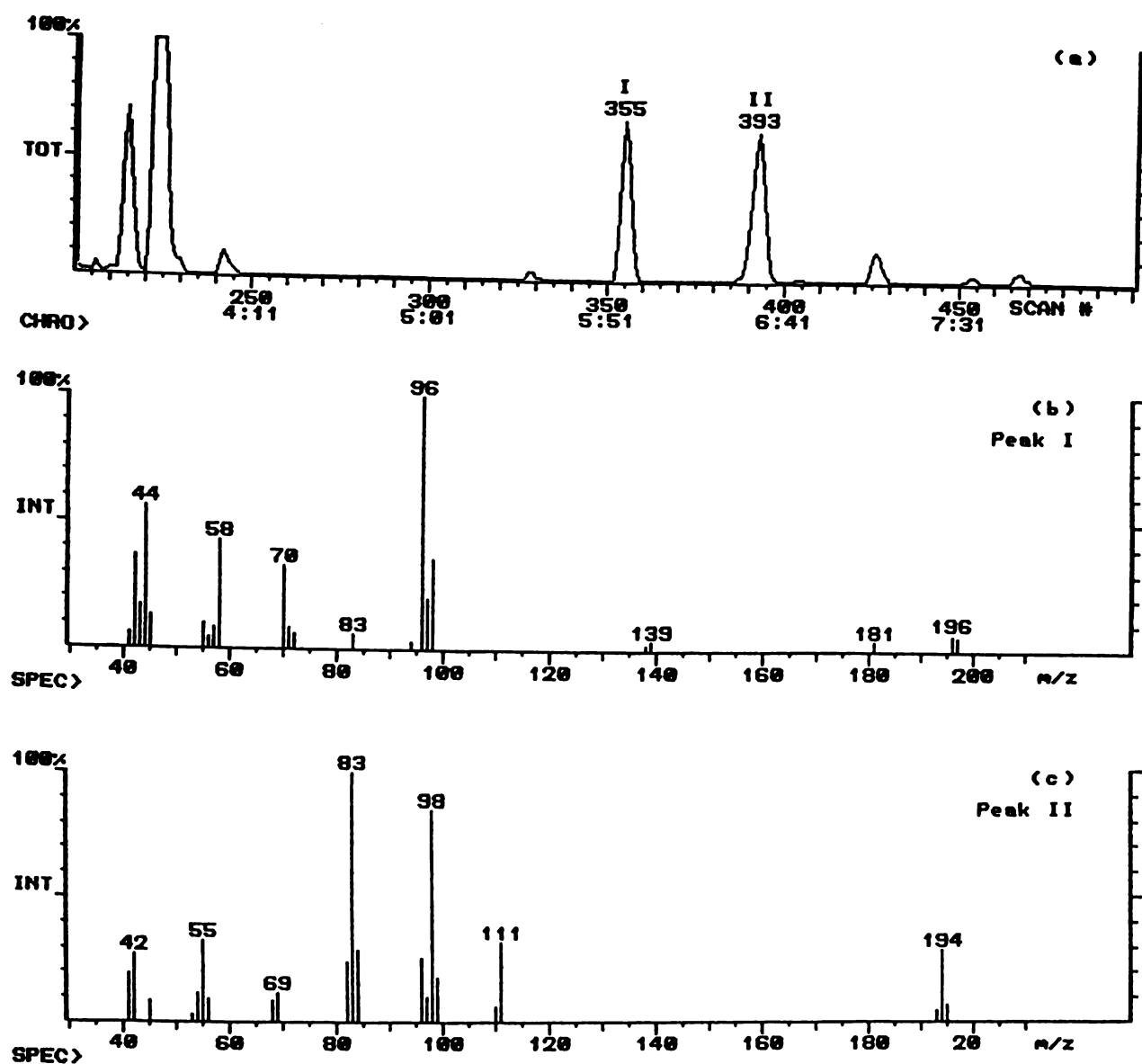


Figure 2. Ion trap detector-gas chromatography of paraquat and diquat reduction products by the treatment with sodium borohydride-nickel (II) chloride. (a) Total ion chromatogram; (b) Mass spectrum of scan number 355, peak I (perhydrogenated paraquat).; and (c) Mass spectrum of scan number 393, peak II (perhydrogenated diquat). GC conditions: Column, DB-17 (0.25 mm i.d. x 30 m, film thickness 0.25 μ m); column temperature, 200°C; split injection, split ratio 50:1; injection temperature, 230°C; carrier gas He, 1 ml/min. ITD conditions: Mass range, 40 - 450; scan rate, 1 scan/s; transfer line temperature, 250°C.

Detection and Evaluation of Cocaine Overdose-Related Deaths

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This report summarizes three case studies describing two types of anomalous laboratory findings associated with cocaine overdose-related deaths. The first case demonstrates postmortem changes in blood cocaine concentrations. The second and third cases demonstrate situations where the Syva EMIT method alone was insufficient for detecting cocaine consumption.

MATERIALS AND METHODS

Urine was analyzed with the Syva EMIT ETS method for benzoylecgonine, 300 ng/mL cutoff (Syva, Mountain View, CA) then by TLC (Toxi-Lab, Irvine, CA). Analyte identification was confirmed by GC/MS with a model 5980 GC and model 5970 MSD fitted with an HP-1, 12 M x 0.20 mm i.d. x 0.33 micron film thickness column (Hewlett-Packard, Palo Alto, CA). Helium flowing at 1.0 mL/min was the carrier. Temperature was programmed to hold 50° for 1 minute, rise to 190° at 25°/minute, hold for 3 minutes, rise to 280° at 10°/minute and hold for 12 minutes.

Blood was extracted by the method of Foerster *et al.* (1978) and analyzed by GC with a model Sigma 2000 GC fitted with a split/splitless injector, flame ionization detector and model 3600 data system (Perkin-Elmer Corp., Norwalk, CT) and an SPB-5, 15 M x 0.32 mm i.d. x 1 micron film thickness column (Supelco, Bellefonte, PA). Helium flowing at 5.0 mL/minute was the carrier. Temperature was programmed similarly to that for GC/MS described above. Quantification was by peak area integration and internal standardization

with mepivacaine; identification confirmation was by GC/MS. Tissues and gastric contents were homogenized prior to extraction and analysis.

CASE STUDIES

In Case 1 a 23-year-old male swallowed an unidentified quantity of cocaine, then proceeded to the home of a friend where he collapsed in convulsions. The subject was pronounced DOA in an emergency room. Cocaine was quantified in urine, blood, and tissues (Table 1). Also identified but not quantified were methlecgonine and benzoylecgonine in urine and methylecgonine and cinnamoyl cocaine in blood.

The increased level of cocaine in postmortem heart blood relative to antemortem blood illustrates the potential problem with evaluating toxic and/or lethal levels of a drug based solely on analysis of postmortem specimen(s). Without the antemortem blood revealing the subject to be toxemic at 2.2 mg/L, one might otherwise conclude that a 13-fold higher cocaine level was responsible for death. Diminished circulation near death allowed heart blood to accumulate the orally consumed cocaine. This phenomenon of site and time-dependent influences of postmortem drug levels has been reviewed recently by Prouty and Anderson (1990).

In Cases 2 and 3 measurement of Benzoylecgonine alone by the Syva EMIT method proved to be insufficient for detecting cocaine consumption. Here, a 27-year-old male (Case 2) and a 37-year-old female (Case 3) were separately found dead at scenes of apparent

self-injections of cocaine. Urine from both cases was negative for benzoylecgonine vase upon EMIT calibrator cutoff criteria (Table 2A). Cocaine was identified in urine from both cases by TLC and/or GC/MS as well as methlecgonine and cinnamoyl cocaine in urine from Case 2. Cocaine was quantified in blood from both cases as well as additional drugs in blood from Case 3 (Table 2B).

In these two cases, rapid death must have preceded the accumulation of urinary benzoylecgonine in quantities detectable by the Syva EMIT method. That cocaine and the analogue, cinnamoyl cocaine and the metabolite, methylecgonine, but no benzoylecgonine were detected in urine illustrates a situation where

urinary benzoylecgonine alone was an insufficient indicator of cocaine consumption.

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Table 1. Cocaine Concentrations in Specimens From a 23-year-old Male Dead From Swallowing a Large, But Unidentified Quantity of Cocaine (Case 1).	
Blood, Antemortem	2.2 mg/L
Blood, Postmortem, Heart	28.5 mg/L
Vitreous Humor	5.0 mg/L
Urine	58.8 mg/L
Kidney	12.0 mg/kg
Liver	12.7 mg/kg
Lung	15.5 mg/kg
Gastric Contents	3180 mg Total

Table 2.		
A. Syva EMIT ETS Rates for Calibrators and Urine Specimens From a 27-Year-Old Male (Case 2) and a 37-Year-Old Female (Case 3) Dead From Apparent Self-Injections of Cocaine.		
	Case 2	Case 3
Negative Calibrator	416	385
300 ng/mL Calibrator	535	514
3,000 ng/mL Calibrator	659	659
Urine Specimens	503	393
B. Concentrations of Cocaine and Other Drugs in Blood From a 27-Year-Old Male (Case 2) and a 37-Year-Old Female (Case 3) Dead From Apparent Self-Injections of Cocaine.		
	Case 2	Case 3
Cocaine	2.3 mg/L	1.7 mg/L
Imipramine	---	Trace
Desipramine	---	1.0 mg/L
Norpropoxyphene	---	0.4 mg/L

Isolation of Toluene from Blood Utilizing a Modified Adsorption/Elution Technique and Identification by Gas Chromatography/Mass Spectrometry (GC/MS)

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Solvent abuse, the deliberate inhalation of organic solvents to produce intoxication, is increasing in popularity. The solvent of choice is usually toluene, which is readily available to persons of any age from most hardware stores. A method is presented which will isolate toluene from blood and allow for direct analysis using GC/MS. This method is derived from a technique commonly used to recover trace amounts of accelerant residues from fire debris (Chrostowski and Holmes 1979).

MATERIALS

Standard 5.25 inch glass pasteur pipet, 50 - 200 mesh activated charcoal, ACS carbon disulfide, standard 20 ml headspace vial with Teflon or foil-lined septum, 5/8 inch 26 ga disposable syringe needles, glass wool, latex tubing, and dry grade nitrogen gas.

EQUIPMENT

Hewlett-Packard 5890 gas chromatograph with Hewlett-Packard 5790 mass selective detector, 12 meter, .20 mm id, .33 micron film HP-1 capillary column.

METHOD

Heat a shallow layer of activated charcoal in a glass

dish in a 500°C oven for about 2 hours to thoroughly activate before using. The charcoal then may be stored in an airtight container for later usage. A small amount of glass wool is placed in a pasteur pipet, followed by 1 inch of charcoal, with another glass wool plug above the charcoal. One mL blood is placed in a headspace vial and capped using a foil or Teflon-lined septum; plain rubber septa can be porous to toluene (Gill *et al.* 1988; Saker *et al.* 1991). Insert one of the disposable needles through the center of the septum and another near the edge at an angle. Inserting the needle at an angle will allow more thorough purging. Attach a 1-inch piece of latex tubing to each needle hub. Attach the charcoal pipet to the center needle and the nitrogen supply to the angled needle; this will induce a more efficient air exchange. Nitrogen pressure should be low—less than 10 psi. The flow can be checked by briefly putting the narrow end of the charcoal pipet in water and observing bubbles. Gently purge the vial with nitrogen for about 1 hour.

Before eluting the toluene from the charcoal, observe the pipet and make sure there is no moisture present. If moisture is present, reattach pipet to nitrogen and purge until dry. Place the pipet in a culture tube and add 2 mL of CS₂. Allow to solvent to flow through completely. Carefully concentrate the CS₂ under nitrogen, without heat until about 100 - 200 µL remains.

Inject 1 μ L into GC/MS under the following conditions: oven temperature program; 40°C isothermal, purge valve initially off, purge on; 0.5 min, solvent delay; 0.95 min, acquisition on; 0.95 min, scan range 40 - 200 amu, injector temperature; 250°C, detector temperature; 280°C. Under these conditions toluene has a retention time of about 1.5 min. The purge on time should be early enough to prevent the solvent tail from overriding the toluene peak.

In conclusion, a method has been devised for the detection of toluene in blood using GC/MS that is simple, rapid, and sensitive. This method could easily be applied to blood analyses for other volatiles. This method is superior to those using headspace GC because GC/MS allows the volatile to be identified more conclusively.

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Information from a Single Hair by X-Ray Fluorescence

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Hair is a valuable substance for forensic purposes because it is often found at the scene of investigation and easily obtained from those suspected of being involved. The concentrations of many elements found in hair are dependent upon the level existing in the blood at the time the hair is formed. Once the hair evolves from the scalp, the concentrations should not change unless caused by external factors. Thus, each point on a hair gives information pertaining to a definite time of the person's life. To get this information, a method of analysis must be sufficiently sensitive to measure the level of an element in a segment of 1 mm.

Chemical methods do not have this sensitivity, and a special x-ray fluorescence instrument (Toribara *et al.* 1982) was built to determine the concentration of mercury along a single hair. This was utilized in a study of the aftermath of a tragic incident where grain seed treated with methylmercury as a fungicide was consumed. Some children born of pregnant women who had eaten the grain were affected and some were not. In order to establish the stage of pregnancy which caused this, the exact time of highest level of mercury in the mother in relation to the birth date was necessary. Figure 1 shows the mercury profile of a single strand of hair from a pregnant Iraqi woman. Minimal effects did not appear until some time after birth such that a precise determination of the time of maximum intake had to be determined at a later date. Knowing the growth rate of the hair it may be seen that the profile shown gives a date within narrow limits.

Since the instrument was programmed to measure simultaneously other elements, it has been used for a number of other applications (Toribara 1987). The appearance of mercury in substantial quantities in some varieties of fish is of concern, and studies of populations such as northern natives of Canada whose diets are

related to seasonal availability of food have produced some interesting results. Figure 2 shows the profiles of the mercury and zinc levels in a strand of hair from an Inuit woman. Note that mercury level is up when the zinc level is low and vice versa. Fish is the chief carrier of mercury, and it has a low zinc content. Red meat has considerably more zinc than fish, and the profiles show a seasonal variation in the diet. Figure 3 shows the profile of a hair from a body found buried under ice for 400 years in northern Alaska. Although the hair was too short to show any yearly profile, it did cover a period where diet change had occurred.

Because this method of analysis is nondestructive, the exact hairs may be scrutinized by another laboratory having similar equipment. In a rape-murder case the attorney for the defendant brought samples for analysis. If the hairs showed different profiles, it would be evidence that they did not come from the same person. Similarity would indicate probably the same source, but other corroborative information is necessary to substantiate this. Table 1 shows the results from 3 hairs as indicated. Of interest is the zinc level which is much lower than would be expected from on the diet available to a city dweller. One reason for a low level of zinc in hair from an adequate diet is alcoholism. The defendant's attorney acknowledged that his client was an alcoholic. The hair taken 4 months later is seen to be completely different, and the reason was that the suspect was in prison during that period with a different diet.

No commercial instrument which could be adapted easily to handle single hairs was available until the recent production of the Kevex Omicron spectrometer. This instrument was designed for analysis of small areas such as those in micro chips. The physical handling of hairs was accomplished by designing a holder for the hairs that fit into the holder supplied

with the instrument. The computer program supplied with the instrument was designed for measuring the surface of metals and was not suitable for the low concentrations of elements in a small segment of single hairs. Special programs have been developed for hair.

The usual preparation of a sample for analysis consists of gently wiping the hair to remove surface dust before mounting the hair in the holder. The holder is placed in the instrument, and the desired program is selected for analysis.

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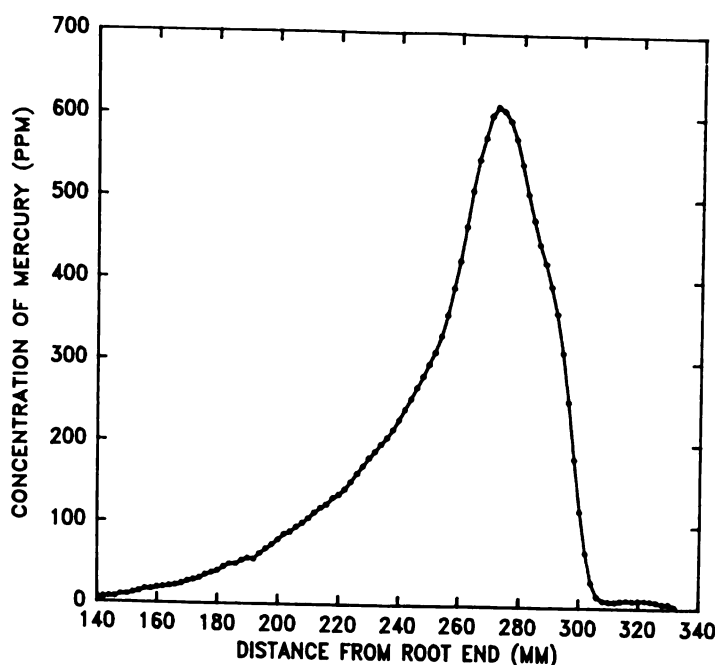


Figure 1. Mercury profile of a single hair from a pregnant Iraqi woman.

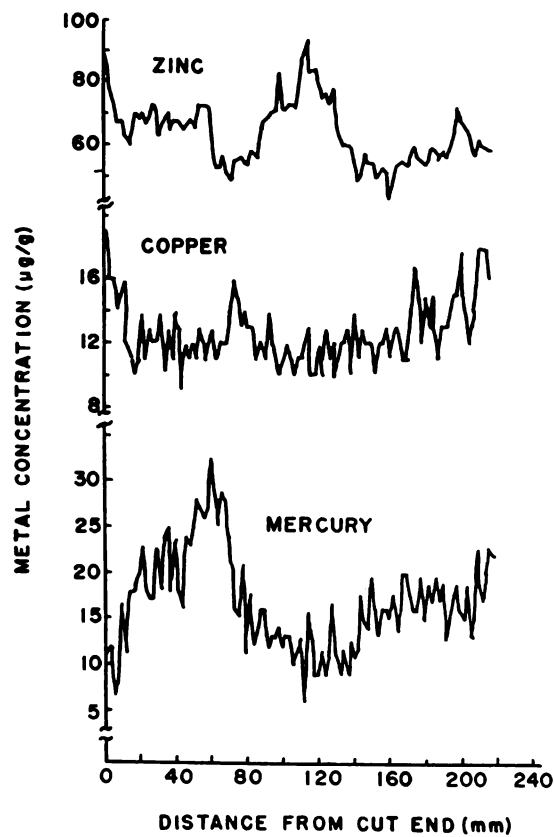


Figure 2. Profiles of hair from a modern Inuit woman (Northern Quebec).

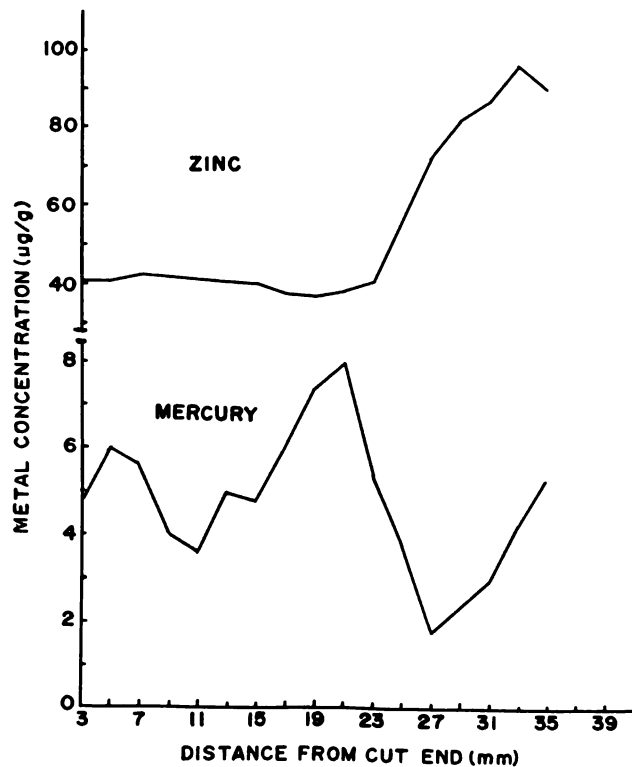


Figure 3. Profiles of hair from body buried in ice in Alaska.

Table 1. Hair Analyses in a Rape/Murder Case.		
	Zn (ppm)	Cu (ppm)
1. Victim	97.0	28.8
	95.7	28.8
2. Suspect (Immediate)	98.8	25.3
	99.3	24.7
3. Suspect (Four Months Later)	70.7	48.1
	71.2	47.7

Use of Postmortem Vitreous Humor for Screening Cocaine Metabolites by Fluorescence Polarization Immunoassay and Further Confirmation by GC/MS

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The urgent need for a rapid automated, sensitive screening for cocaine metabolites makes vitreous humor a practical alternative when urine or other body fluids are unsuitable or not available. Vitreous humor requires no sample pretreatment and the small amount collected is sufficient for extraction and its further identification and quantitation by GC/MS.

MATERIALS AND METHODS

The Abbott TDx (FPIA) instrument was calibrated with urine calibrators supplied by Abbott. The instrument was set to report numerical semiquantitative results of benzoylecgonine present in vitreous humor. After TDx screening, vitreous humor or blood from 92 actual cases were extracted using Clean Screen DAU extraction procedure. Extraction columns (130 mg), fritted reservoirs (8 mL) adapters, and a vacuum manifold system were obtained from Worldwide Monitoring Corporation (Horsham, PA). One mL of vitreous humor, blood standards, and controls were used. Three mL of deionized water was added, vortexed, and then centrifuged. The samples were decanted into clean dry test tubes. One mL of phosphate buffer (pH 7) was added to each sample. One mL of 0.5 µg/mL cyclizine (Sigma Chemical Company, St. Louis, MO) as internal standard was added to the column followed by samples previously prepared. Drugs were eluted with a mixture of methylene chloride:isopropanol: ammonium hydroxide (40:10:1), (Fisher Scientific, Fair Lawn, NJ). The eluate was dried under nitrogen flow,

reconstituted with 50 µL BSTFA + 1% TMCS (Alltech Associates Inc., Deerfield, IL) and heated at 60°C for 15 minutes to form the trimethylsilyl derivative of benzoylecgonine. One µL of remaining solution was injected into the GC/MS (Hewlett Packard Model 5970B MSD) equipped with a 7673A automatic liquid sampler. The column is a HP-1 methyl-silicone capillary column 12 m x 0.20 mm i.d, 0.33 µm film thickness, temperature was maintained at 165°C for 1 minute and then programmed at 15 minutes to 280°C, the injector port temperature was 220°C and the transfer line temperature was 290°C. For quantitative analysis, the MSD was operated in the selective ion monitoring (SIM) mode. The following ions were monitored for each compound: Cocaine m/z 82.20, 105.20, (182.20), and 303.20 (R_t = 6.22 min), Benzoylecgonine m/z (240.10), 256.10, 346.10, 361.10 (R_t = 6.72 min), Cyclizine m/z 99.05, 167.15, (194.10), 266.20 (R_t = 5.14 min). The ions noted in parenthesis were used for quantitation. Calculation of the unknown samples is based on a response factor derived from a standard curve. Identification of the drugs is done by comparison of the unknown specimen spectrum with a library of reference spectra developed with known standards.

RESULTS AND DISCUSSION

Vitreous humor from actual autopsy cases were screened by TDx and either the blood or vitreous humor was confirmed negative or positive by GC/MS. Semiquantitative benzoylecgonine results obtained from FPIA that were lower than 0.03 µg/mL were not found

positive by GC/MS. The limit of detection for cocaine and benzoylecgonine was found to be 0.02 µg/mL by GC/MS. TDx numerical values that report higher or lower concentrations of cocaine metabolites in vitreous humor will confirm higher or lower quantitative concentrations respectively in either blood or vitreous humor by GC/MS. This showed excellent correlation

between the two methods (see Table 1). Both the TDx system and the GC/MS quantitation and identification use a completely automated procedure using a small amount of specimen. The use of vitreous humor is a practical alternative to routine cocaine metabolites screening in forensic toxicology.

Table 1. GC/MS Confirmation of Cocaine and Benzoylcegonine on Representative Postmortem Samples of Vitreous Humor and/or Blood After Screening Vitreous Humor by TDx.

Case Number	TDx	GC/MS			
	Vitreous Humor	Vitreous Humor		Blood	
	Benzoylecgonine $\mu\text{g/mL}$	Cocaine $\mu\text{g/mL}$	Benzoylecgonine $\mu\text{g/mL}$	Cocaine $\mu\text{g/mL}$	Benzoylecgonine $\mu\text{g/mL}$
92 - 1	0.03	ND	ND	NF	0.05
92 - 2	0.03	ND	ND	NF	0.02
92 - 3	0.46	0.47	1.53	NA	NA
92 - 4	0.71	0.08	0.52	0.23	0.93
92 - 5	0.00	NF	NF	NF	NF
92 - 6	0.23	NF	0.20	0.46	0.64
92 - 7	0.02	NF	NF	NF	NF
92 - 8	4.98	0.02	3.23	NA	NA
92 - 9	1.82	0.89	0.82	0.30	1.49
92 - 10	0.13	NF	0.14	NF	NF
92 - 11	0.02	NF	NF	NF	NF
92 - 12	0.11	NF	0.14	0.19	0.23
92 - 13	0.51	0.09	0.66	0.09	0.60
92 - 14	0.22	0.05	0.23	NF	0.12
92 - 15	0.06	0.04	0.07	NF	0.11
92 - 16	0.09	0.03	0.06	NF	0.03
92 - 17	0.49	0.06	0.32	0.04	0.63
92 - 18	0.01	NF	NF	NF	NF
92 - 19	2.17	0.99	2.07	0.82	5.30
92 - 20	0.39	1.34	0.39	0.34	0.76
92 - 21	0.20	0.06	0.19	0.07	0.22
92 - 22	0.13	0.63	0.07	0.44	1.05
92 - 23	1.49	2.60	1.02	0.09	0.29
92 - 24	0.33	ND	ND	0.02	0.32
92 - 25	2.90	ND	ND	0.73	2.60
92 - 26	0.03	ND	ND	NF	0.05
92 - 27	0.03	ND	ND	NF	0.02
92 - 28	0.07	ND	ND	0.03	0.29

NF - Not Found NA - Not Available
 ND - Not Determined

Simple Modification of a Single Hewlett-Packard 5970 Mass Selective Detector Allowing for Routine Forensic Analyses Utilizing Dual Capillary Columns

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Pressed by an increasing laboratory workload, mounting financial constraints, and the necessity to routinely characterize pharmaceuticals and extracts from various biological matrices, we have simply and successfully modified a Hewlett-Packard (HP) 5970 Electron Impact Mass Selective Detector (MSD), to allow for dual capillary columns to be interfaced into a single MSD. The instrument has been utilized in the analysis of both underivatized drug analytes by linear scan analysis and derivatized [trimethyl silyl (MSTFA)] compounds using selected ion monitoring (SIM) techniques.

Three dual column approaches were evaluated, glass-polymide "Y" connectors (GPYC) (Figure 1), zero-dead volume "Splitter Unions" (ZDVSU) (Figure 2) (Hewlett-Packard, Avondale, PA) and the dual column guide tube insertion technique (DCGTI) (Figure 3). The optimal DCGTI approach makes use of a larger guide tube assembly (Hewlett-Packard) and a "hand-drilled" two-holed ferrule for the simultaneous introduction of dual (DB-5, 5%-phenyl, 0.20 mm i.d. x 30 m, 0.25- μ m film thickness, J&W Scientific) capillary columns directly into a single ion source. This

approach permits selected single column analysis for specific analyte residues.

Initially examined were the GPYC column connectors. Despite achieving a technically correct seal of the "Y" connector, unacceptable atmospheric background was present due to the inherent need and use of a high system vacuum proximal to the connector. This allowed for outside atmosphere to be pulled into the source in spite of the initial seal.

ZDVSU's were next examined. We incorporated a ZDVSU (Hewlett-Packard) into our GC/MS system and it proved initially successful, as have other laboratories, as an acceptable means of dual column utilization with a single GC/MSD. However, in making adjustments to the capillary head pressure to obtain a linear carrier velocity of 35 - 40 cm/s through one of the two columns, flow problems were encountered. These were determined to be due to difficulties in achieving and maintaining proper ferrule alignment when securing the columns to the splitter union. In addition, this alignment periodically led to the introduction of atmosphere into the source.

Using the DCGTI approach, problems with the maintenance of a high negative vacuum and atmospheric leakage into the source were found to be minimal. Moreover, similar molecular fragmentation in the 40 - 550 amu range, good analyte sensitivity and minimization of column to detector transfer problems were demonstrated. Finally, problems with detector wear (time to source cleaning) and implicit SOP's for the GC/MSD system as delineated by (HP) (Cuyahoga County Coroner's Office 1991) and our laboratory protocol (Hewlett-Packard 1986) were minimal. Initially when securing the columns with this approach, we found that some commercially available 0.35 mm two-holed ferrules led to excess atmospheric leakage at the SGE GC/MSD interface nut connection. By drilling our own ferrules, (Scientific Glass & Engineering, Austin, Texas. #GVf2/003) using #80, 0.35 mm drill bits (Cleveland Twist Drill, Cleveland, OH) we were able to achieve a superior reduction in atmospheric background. The slightly higher graphite content of the ferrules allowed for less torquing of the SGE interface nut and prevented column sheering at this junction.

Instrumental sensitivity experiments were performed and signal-to-noise values were obtained for the ions of Methyl Sterate and were consistent with manufacturer specifications (Perkins 1987). Autotune specifications for the instrument were within normal acceptance criteria. The DCGTI technique provides satisfactory responses in all area as compared to the more traditional single column/single detector approach.

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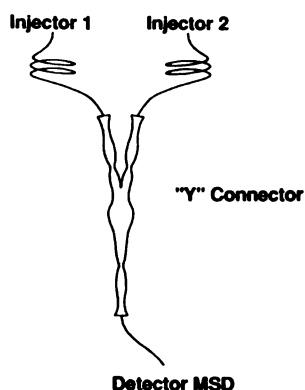


Figure 1.

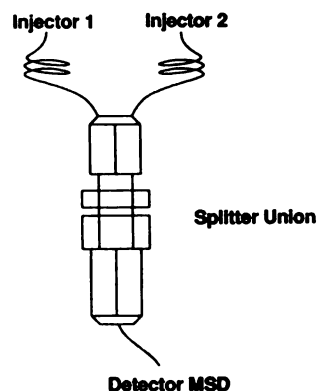


Figure 2.

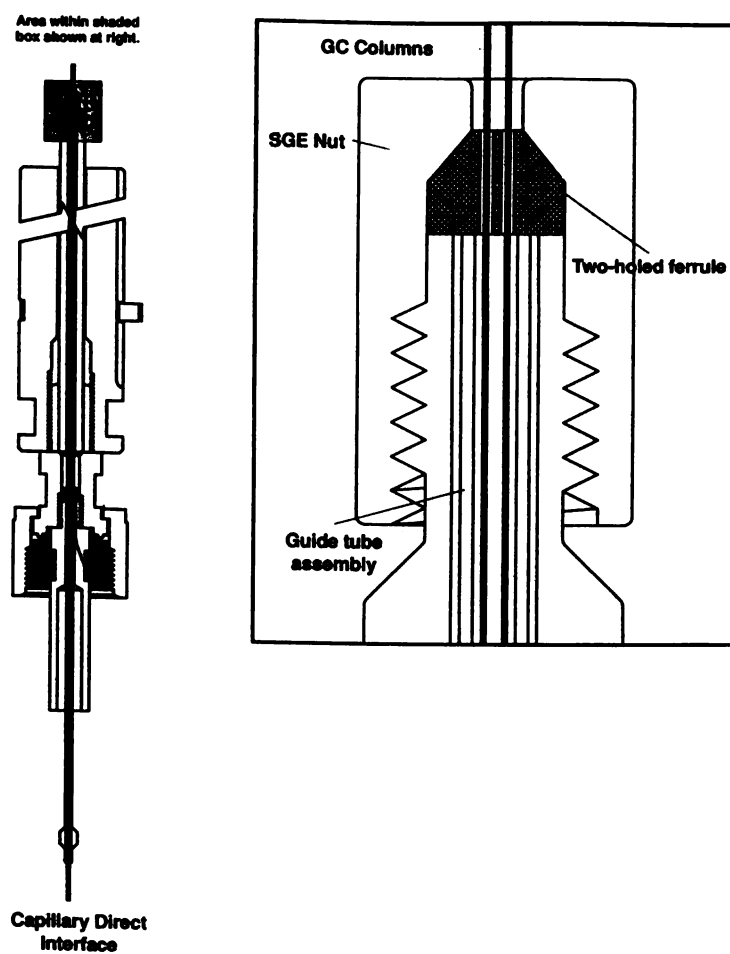


Figure 3.

A Class-Independent Drug Screen in Forensic Toxicology Using a Photodiode Array Detector

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A challenging task in clinical and forensic toxicology is the identification of unknown substances in biological tissues. We have developed a rapid gradient elution HPLC method using a photodiode array detector for a toxicological screen of a large number of drugs and poisons in postmortem blood to complement our capillary GC screen for basic substances.

MATERIALS AND METHODS

The liquid chromatograph consisted of two Model 6AD constant flow pumps, a gradient mixing chamber, Model SIL-6B autoinjector, system controller (SCL-6B), Model SPD-M6A photodiode array detector (Shimadzu Instruments) and a PC-AD computer (SAMSUNG S550). A backup tape drive, an additional Model SPD 6AV ultraviolet detector operating at 230 nm and a Model CR-4A integrator/plotter (Shimadzu Instruments) was connected in series after the photodiode array detector. The photodiode array detector was operated in a 1 nm band-pass mode monitoring light from 195 - 650 nm. Display wavelengths were usually 230 nm and 214 nm. The analytical column was a 5 μ m particle-size spherisorb S5 ODS-2 column (Phase Separations) (15 cm long x 3.8 mm I.D.) protected by a NOVAPAK C18 guard column (Waters Associates). The mobile phases consisted of 10% acetonitrile in 10 mM potassium phosphate buffer, pH 3.1 (Pump A) and 60% acetonitrile in 10 mM potassium phosphate buffer, pH 3.1 (Pump B). Gradient conditions used were: isocratic at 0% pump B for 1 minute, linear gradient to 50% pump B over 5 minutes, isocratic at 50% pump B for 20 minutes, then a linear gradient to 100% pump B over 10 minutes, followed by a hold at 100% pump B

for 10 minutes. Total run time was 46 minutes and the total flow rate was 1.0 ml/minute.

Blood (fresh or postmortem), standards or controls (250 μ l) were added to polypropylene micro-extraction tubes (1.5 ml capacity) followed by 250 μ l of acetonitrile containing 80 mg/l p-tolyl-phenyl-hydantoin as internal standard. Samples were properly mixed, allowed to stand for 10 minutes, and centrifuged in a micro-centrifuge at the highest speed (approx. 10,000 g. max) for 10 minutes. An aliquot of the supernatant, usually 20 μ l, was injected into the HPLC.

RESULTS AND DISCUSSION

Assays for postmortem screening of blood samples were performed in batches incorporating at least five standards, each containing several analytes, and quality control samples. Standards were run with each batch to minimize variations in RRT from one run to another. Tentative identification of drugs was based on the RRT, with an allowable range of ± 0.05 of the library entries and the extracted standards. The UV spectrum of the unknown peak was then called up onto the VDU screen. Assessment of the compound's UV was further assisted by a library match facility on the computer software. A UV spectra match of 95% was taken as a confirmation although verification by a second method of analysis, which also formally quantified the concentration of the substance, was usually performed.

The RRT of approximately 100 drugs and poisons are summarized in Table 1. The earliest eluting drugs allopurinol, metformin, paracetamol, and theophylline are separated from the solvent front and can be easily identified by their characteristic UV spectra. Where

components coelute with each other such as phenobarbital and ethacrynic acid (RRT 0.64) characteristic UV spectra permit ready identification. Matches of better than 95% were generally obtained. There was, however, some problem in identifying coeluting barbiturates such as pentobarbital and hexobarbital because of their similar UV spectra.

This method has been successfully used in our laboratory to screen over 1,000 postmortem blood specimens. Paracetamol was the most common drug detected, followed by carbamazepine, theophylline, and salicylate. Barbiturate poisoning accounted for 11 cases, and 4 deaths involved theophylline. An advantage of this method is that no selective extraction procedure was used to clean up the blood sample, thus allowing any substance, if present in sufficiently high amounts, to be detected. This approach has enabled detection of drugs other than the acidic and neutral drugs previously mentioned. Benzodiazepines such as oxazepam, diazepam, nordiazepam, lorazepam, and temazepam are detected as well as antidepressants such as dothiepin, imipramine, amitriptyline, trimipramine, and doxepin

and drugs such as *propranolol* and verapamil, particularly when present in concentrations at the upper end of their therapeutic range, or above.

Three examples of postmortem cases (see Figure 1), demonstrate the versatility of this methodological approach. The first case (A), an overdose of verapamil, shows the pattern of verapamil metabolites and other drugs present such as thiopentone, pentobarbitone, and frusemide. In the second case (B), a therapeutic concentration of paracetamol is shown together with carbamazepine and its metabolites. The third case, example (C), is an overdose of doxepin, showing the metabolite pattern and the other drugs phenytoin and thiopentone both in therapeutic concentrations.

This method may be used in both a clinical toxicology setting to establish the presence and concentration of drugs in acute poisonings, as well as in a forensic toxicology setting where the presence of drugs is often required to help reconstruct the events leading up to death.

Table 1. Relative Retention Times of Selected Drugs.*			
Allopurinol	0.12	Methyl Phenobarbital	0.84
Metformin	0.21	Methosuximide	0.86
Paracetamol	0.23	Oxazepam	0.86
Azetazolamide	0.24	Glutethimide	0.87
Metronidazole	0.26	Oxprenolol	0.89
Theophylline	0.27	Quinalbarbitone	0.90
Hydrochlorothiazide	0.30	Labetolol	0.90
Chlorothiazide	0.38	Lorazepam	0.91
Caffeine	0.44	Diflunisal Metabolite	0.93
Cimetidine	0.46	Chlormethiazole	0.94
Ethosuximide	0.47	Chlorpropamide	0.95
Methotrexate	0.48	Piroxicam	1.00
Picloram	0.49	Sulindac	1.06
Primidone	0.54	2,4-Dichlorophenoxyacetic Acid	1.06
Salicylic Acid Metabolite	0.53	Desalkyl Flurazepam	1.11
Baclofen	0.55	Tolbutamide	1.11
Phenylmethyl Barbital	0.56	Thiopental	1.13
Sulthiame	0.56	Glipizide	1.13
Ranitidine	0.57	Temazepam	1.14
Naproxen Metabolite	0.58	Propranolol	1.14
Dextropropoxyphene Metabolite	0.59	Nordiazepam	1.17
Pindolol	0.60	Alprenolol	1.18
Chlorthalidone	0.60	Ketoprofen	1.26
Acetyl Salicylate	0.61	Cyclopenthiiazide	1.26
Sulphamethoxazole	0.61	Bendrofluazide	1.26
Phenobarbital	0.64	Naproxen	1.28
Ethacrynic Acid	0.64	Gliclazide	1.54
Salicylic Acid	0.65	Diazepam	1.58
Carbamazepine Epoxide	0.65	Nordoxepin	1.60
Meclobamide	0.66	Probenecid	1.69
Clopamide	0.66	Warfarin	1.80
Diflunisal Metabolite	0.67	Diflunisal	1.91
Tenoxicam	0.67	Fenoprofen	1.97
Butobarbital	0.67	Doxepin	1.98
Phenacetin	0.67	Norfluoxetine	2.15
Trimethoprim	0.67	Diclofenac	2.18
Metoprolol	0.70	Indomethacin	2.18
Phensuximide	0.71	Ibuprofen	2.21
Doxepin Metabolite	0.73	Glibenclamide	2.23
Methyclothiazide	0.74	Dothiepin	2.25
Pentobarbital	0.79	Phenylbutazone	2.30
Hexobarbital	0.79	Verapamil	2.33
Quinine	0.79	Imipramine	2.34
Frusemide	0.82	Mefenamic Acid	2.42
Amylobarbitol	0.82	Amitriptyline	2.47
Phenytoin	0.82	Trimipramine	2.49
Carbamazepine	0.84		
* MPPH (Internal Standard) = 1.0			

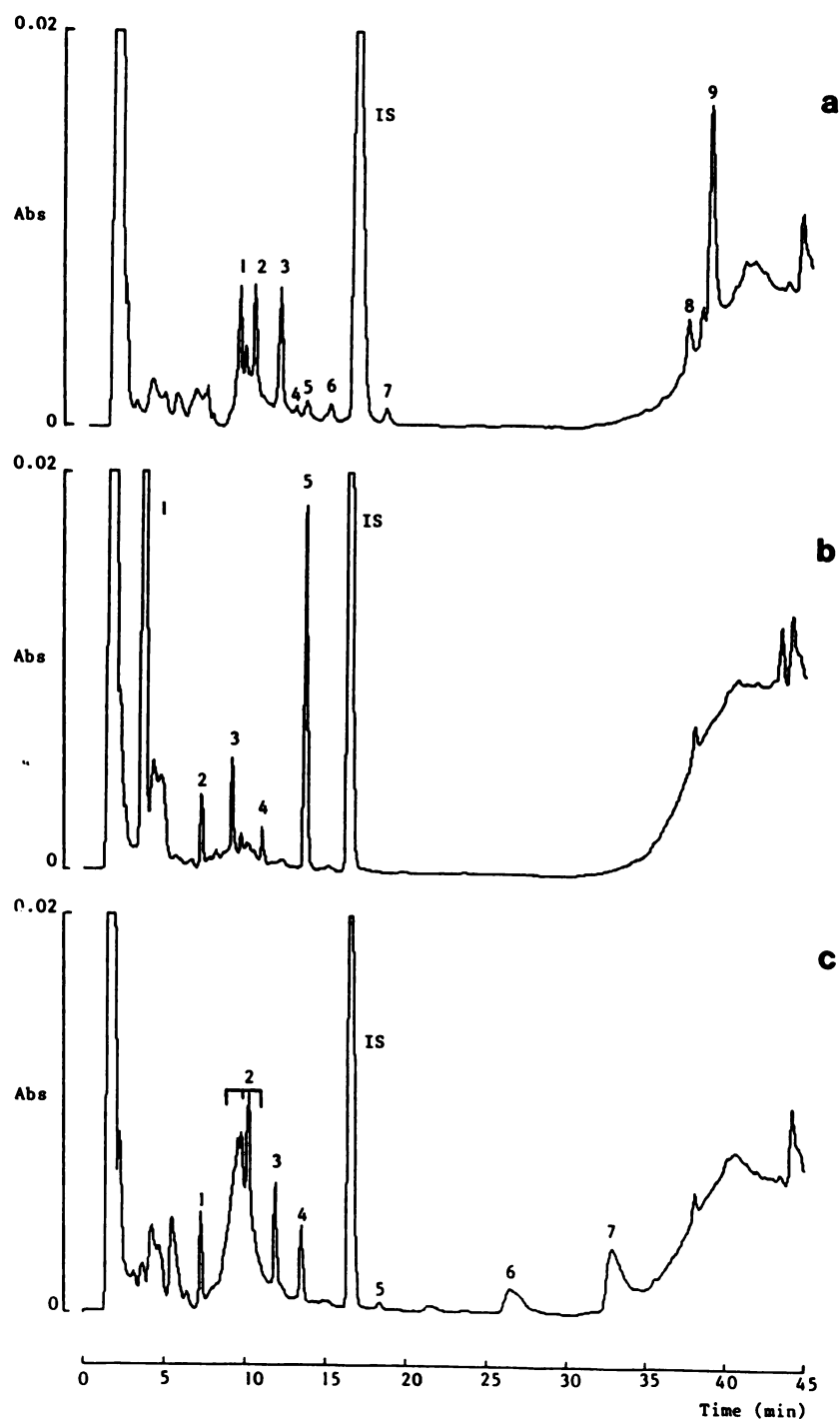


Figure 1. Chromatograms obtained from postmortem blood of: (A) 1. Verapamil metabolite; 2. Endogenous peak; 3. Verapamil metabolite; 4. Pentobarbitone (2 mg/l); 6. Verapamil metabolite; 7. Thiopentone (2 mg/l); 8. Verapamil metabolite; 9. Verapamil (12 mg/l); (B) 1. Paracetamol (24 mg/l); 2. Caffeine (3 mg/l); 3. Carbamazepine metabolite; 4. Carbamazepine-10, 11-epoxide (0.5 mg/l); 5. Carbamazepine (10 mg/l); and (C) 1. Caffeine (4 mg/l); 2. Endogenous peaks; 3. Doxepin metabolite; 4. Phenytoin (8 mg/l); 5. Thiopentone (1 mg/l); 6. Nordoxepin; and 7. Doxepin (5 mg/l).

Occupational Exposure to Cocaine Involving Crime Laboratory Personnel

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The possibility of exposure to cocaine as a result of analyzing the substance or handling material contaminated with cocaine has been a major concern of crime laboratory personnel. Several different work environments and simulated situations were examined to assess the likelihood of this type of exposure occurring. Urine specimens were collected and analyzed for cocaine and benzoylecgonine using a SYVA ETS system, an automated analyzer for drugs of abuse in urine. Subsequently, each urine specimen was then prepared for GC/MS using a solid phase extraction of cocaine and benzoylecgonine utilizing the Zymate Laboratory Automation System (Zymark Corporation) and solid phase extraction columns. The extraction was followed by the formation of the trimethylsilyl (TMS) derivatives. GC/MS was performed using Hewlett-Packard 5890/5970A GC/mass selective detector (MSD), equipped with a 12 m HP-1 capillary column and utilizing helium as carrier gas at 1.0 mL/min. at 200°C oven temperature with a split ratio of 20:1. The system purge was 2.0 mL/minute. The injector and interface temperatures were 250°C and 280°C, respectively. The temperature program was 160°C to 260°C at 20°C/minute. Urine specimens of laboratory management personnel not working with drug samples showed no trace of cocaine or benzoylecgonine. A urinary benzoylecgonine level

of 227 ng/mL was found in the specimen from one narcotics criminalist who was working on a routine case containing 2 kilos of cocaine hydrochloride in the Narcotics Laboratory. A maximal urinary benzoylecgonine concentration of 1570 ng/mL was determined in the urine specimen from one narcotics criminalist who was sampling a case containing 50 kilos of cocaine hydrochloride over a 3-hour time period. Our study has shown that exposure to cocaine involving crime lab personnel does exist. The levels of exposure, in most instances, would be so low that they would not produce positive results for benzoylecgonine by immunoassay at a 300 ng/mL cutoff value. Low levels of benzoylecgonine could, however, be detected by GC/MS. We also learned that prolonged exposure to airborne cocaine dust could produce significant urinary cocaine and benzoylecgonine concentrations. This study emphasizes the need to use caution when dealing with cocaine evidence, especially when working on large cocaine seizure cases. Limiting the generation of airborne cocaine dust has been proven effective in reducing the levels of exposure. It is also important to point out that a criminalist can significantly limit the amounts of exposure to cocaine by wearing face mask, latex gloves, safety goggles/glasses, and his/her lab coat when actively analyzing cocaine evidence.

Blood and Other Tissue Cocaethylene Concentrations in Coroner's Cases

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Cocaethylene has been found in postmortem blood and other tissues in coroner's cases which tested positive for both cocaine and ethanol (Hearn *et al.* 1991). In order to evaluate the frequency and concentration of cocaethylene in our cases, cocaethylene has been included as part of the routine confirmation procedure for all coroner's blood samples which tested positive for benzoylecgonine with the Roche Abuscreen® kit since March 1991. All of the cases were also tested for ethanol. Even if no alcohol was detected, cocaethylene was included in the confirmation procedure. In cases where cocaine intoxication may have contributed directly to the cause of death, brain and liver cocaethylene concentrations were determined in addition to blood levels.

To analyze for cocaethylene, the same method that has been used for cocaine and benzoylecgonine was used to simultaneously analyze for all three compounds (Spiehler and Reed 1985). Cocaine- d_3 and benzoylecgonine- d_3 were added to 0.5 ml blood or 0.5 g tissue (1 g of a 1:1 homogenate) in disposable screw-cap tubes. One ml of phosphate buffer and 1 g of NaCl were added, and the samples vortexed. The samples were extracted with 5 ml of $CHCl_3$:Isopropanol (10:1). The organic layer was filtered through glass wool into another disposable screw-cap tube and evaporated to dryness. Five drops of dimethylformamide and 5 drops of dimethylformamide di-propyl acetal were added to each tube. The tubes were incubated for 30 minutes at 130°C to derivatize the benzoylecgonine. Five ml BuCl and 3 ml 0.5 N H_2SO_4 were added to each tube. After extraction, the organic layer was discarded. A second

wash with 5 ml of BuCl was also discarded. The aqueous layer was saturated with solid $NaHCO_3$ and extracted with 5 ml BuCl. The BuCl was then transferred to a disposable centrifuge tube and evaporated to dryness. The residue was reconstituted in 4 or less drops of $CHCl_3$:EtOH (1:1) and transferred to the autosampler of a GC/MS equipped with a 25 meter HP-1 column. Initial oven temperature was 180°C and then ramped immediately at 70°C/minute to 290°C. The following ions were monitored in the SIM mode: 182, 185, 303, and 306 for cocaine and cocaine- d_3 ; 210, 213, 331, and 334 for benzoylecgonine and benzoylecgonine- d_3 ; 185, 196, and 317 for cocaethylene. (At this time cocaine- d_3 was used for cocaethylene quantitations. Cocaethylene- d_3 is now available from Radian Corp., Austin, TX.) Twenty ng/ml was our cutoff for all compounds in the GC/MS confirmation.

Cocaethylene was detected in 32 of 40 blood samples that contained alcohol. Three of these cases did not contain any parent cocaine in the blood at the time of testing, although it was detected in the brain and/or liver. In eight cases where alcohol and cocaine were present in the blood, no cocaethylene was detected in the blood, but in one of these cases it was detected in the liver. In five cases cocaethylene was detected in the blood, even though the blood alcohol level was negative. In four of these cases, ethanol abuse or prior drinking was noted by the investigator.

Negative blood alcohol results and/or negative blood cocaine results do not rule out the possibility of cocaethylene being detected in other tissues.

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Table 1. Summary of Results

Tissue	Range ($\mu\text{g/ml}$ or $\mu\text{g/g}$)		Median ($\mu\text{g/ml}$ or $\mu\text{g/g}$)	
	Cocaine	Cocaethylene	Cocaine	Cocaethylene
Blood	Neg - 3.7	0.021 - 0.26	0.16	0.066
Brain	0.070 - 19	0.022 - 0.66	1.9	0.19
Liver	Neg - 33	0.040 - 1.4	0.35	0.30

Identification and Quantitation of Salbutamol (Ventolin Inhaler) from Postmortem Blood in a Suspected Overdose

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The use of salbutamol (Ventolin) for the relief of bronchial spasms in asthmatic individuals is common. In this instance a three-year-old female died shortly after receiving repeated doses from a Ventolin inhaler. The laboratory was asked to identify and quantitate the salbutamol in the deceased's body fluids.

A method was developed which is a substantially modified version of work by Leferink, *et al.* (Journal of Chromatography, 1982, 229:217-221). The procedure in this clinical study required many changes to make it suitable for this application on postmortem whole blood. Modifications were also called for due to instrumentation differences and the lack of deuterated compounds for internal standards.

In the original paper, deuterated salbutamol was used as an internal standard. This was found to be cost-prohibitive for our laboratory and another compound, terbutaline, was selected. Due to its structural similarity to salbutamol, it was hoped this compound would yield similar extraction and derivitization characteristics.

Standards were prepared in the range of 100 - 300 ng/ml using blank postmortem whole blood. A 1 ml sample size was used with sample and standards being prepared by the addition of an aqueous internal standard solution and 1 ml of a 0.1 M pH 7.6 phosphate buffer.

The column clean-up and elution procedure used

followed the reference with two variations. The first was a hexane wash substituted for the water wash after sample loading. This was done to improve the removal of contaminants from the postmortem blood. The other was a change in elution solvent to a 80/20 acetonitrile/methanol mix which improved the yield and shortened the dry down step of the procedure.

After dry down, the residue was subjected to a partition step to further clean up the eluent. This was done with the addition of 0.5 ml of acetonitrile and 1 ml of hexane followed by vortexing and centrifugation. The hexane was discarded and the acetonitrile taken to dryness.

For derivitization the residue was dissolved in 50 μ l of BSTFA and incubated at 80° for 15 minutes.

Instrumentation was a Hewlett-Packard 5970A MSD coupled to a Hewlett-Packard 5790 GC with a 15 meter by 0.25 mm ID J&W DB-1 capillary column. The oven profile started at 200° with a 1-minute hold, programmed at 10° per minute to 295°. The MSD was used in the selected ion monitoring mode using three ions for each compound of interest. Since the laboratory's MSD was not equipped for chemical ionization, other ions from those of the first study had to be chosen. For salbutamol these were 369, 370, and 371 amu. For terbutaline the ions were 356, 357, and 358 amu. Using these parameters the retention time for salbutamol was 2.57 minutes and for terbutaline,

A curve generated by the method above gave a correlation coefficient of 0.999 and the salbutamol was shown in the sample to be 137 ng/ml. The sensitivity level of this procedure was determined to be approximately 10 ng/ml. Further, sensitivity could have been realized by using a larger than 1 ml sample size or

by altering the MSD *parameters* to monitor only one ion for each compound as *done* in the original study. In this case it was thought *prudent* to utilize three ions in order to compare ion ratios to *known standards* for identification purposes.

Robotic Analysis of Whole Blood and Postmortem Tissues for Cocaine, Benzoylecgonine, and Opiates Using Solid Phase Extraction

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In March of 1990, the Los Angeles County Coroner's Office purchased a Zymate II robotic arm from Zymark (Hopkinton, MA) in an effort to automate an increasing number of cocaine confirmations. The original Bond Elute Certify chemistry (Varian/Analytichem Bond Elute Certify Methodology) was modified to use a Strong Caton Exchange (SCX) column and a method similar to Logan *et al.* (1990). The resulting procedure also proved to be useful for the analysis of free opiates in whole blood and tissues.

MATERIALS/EQUIPMENT

All acids/bases are reagent grade. All solvents are HPLC grade. Only deionized water is used for reagent preparation. Extraction columns were from Varian (Harbor City, CA). Deuterated (N-D3) cocaine(coc)/benzoylecgonine (BE)/codeine/morphine were from Sigma Chemical Company (St. Louis, MO). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) is from Huls (Piscataway, NJ). Trimethylamine (23-25 wt.%) is from Aldrich Chemical Company (Milwaukee, WI). The Zymate II robot has 15 "pysections" circling a central arm which performs such functions as vortexing, reagent dispensing, pipetting, centrifugation, autosampler vial manipulation, and solid-phase extraction. Final analyses were performed on a Hewlett-Packard 5890GC/5970MSD.

METHOD

After the robot weighs/records the empty 16 x 100 test tubes, 1.5 ml of whole blood or tissue homogenate (50:50 w/w water) is added to each tube and the tube is reweighed. A gravimetric determination of the delivered sample is made and the amount of internal standard (I.S.) then added is adjusted accordingly. After mixing, the samples are equilibrated for 20 minutes and then the protein is denatured with 4 ml of 5% trichloroacetic acid (w/v in water). The samples are vortexed for 90 seconds and centrifuged for 20 minutes at 2800 RPM.

The SCX column is activated with 3 ml of methanol followed by 3 ml of 12 mM HCl. The supernatant from the centrifuged tube is poured onto the column and the sample is loaded onto the resin by means of syringe (flow rate < 2.5 ml/min). The column is washed with: 1 ml 12 mM HCl, 2 ml 12 mM H₃PO₄, 2 ml 70 mM glacial acetic acid, and 3 ml of 2-propanol. After 30 seconds of drying, the drugs are eluted with 2 ml of elution solvent (2% trimethylamine/20% 2-propanol/ 78% methylene chloride v/v/v). The eluent is blown down at 45°C under dry air and reconstituted with 100 µl each of acetonitrile and MSTFA. The solvent/derivatization reagent is transferred to a GC/MS autosampler vial, crimp-capped and heated at 75°C for 20 minutes.

DISCUSSION

Standards and control samples are prepared in NaF preserved porcine blood (pH >6, <7) at levels of: 50, 100, and 1,000 ng/ml coc and Be and 4,000 ng/ml coc and BE separately to monitor hydrolysis of coc to BE (<3%). The I.S. (1.6 ug/ml) is spiked at 200 ng/ml equivalent concentrations. A two point line (100/1,000 ng/ml) is used to calculate the control and specimen quantitations. Triple-ion monitoring and retention times of the ion chromatograms are used for identification. Limits of quantitation currently used are 30 ng/ml for coc/BE. Codeine and morphine quantitation and identifications are handled in an analogous manner. Absolute recoveries for a run of coroner cases (N=18) using the duterated I.S. for reference averaged 85% for cocaine, 93% for BE, 78% for codeine, and 52% for morphine. The robot's I.S./sample accuracy showed a coefficient of variation of

0.4%. Robotic times are *2.3 hours for the first sample* and 20 minutes for each sample thereafter. The cocaine confirmation procedure has *been operating well* for over 18 months and the free opiate version has been used for over 12 months.

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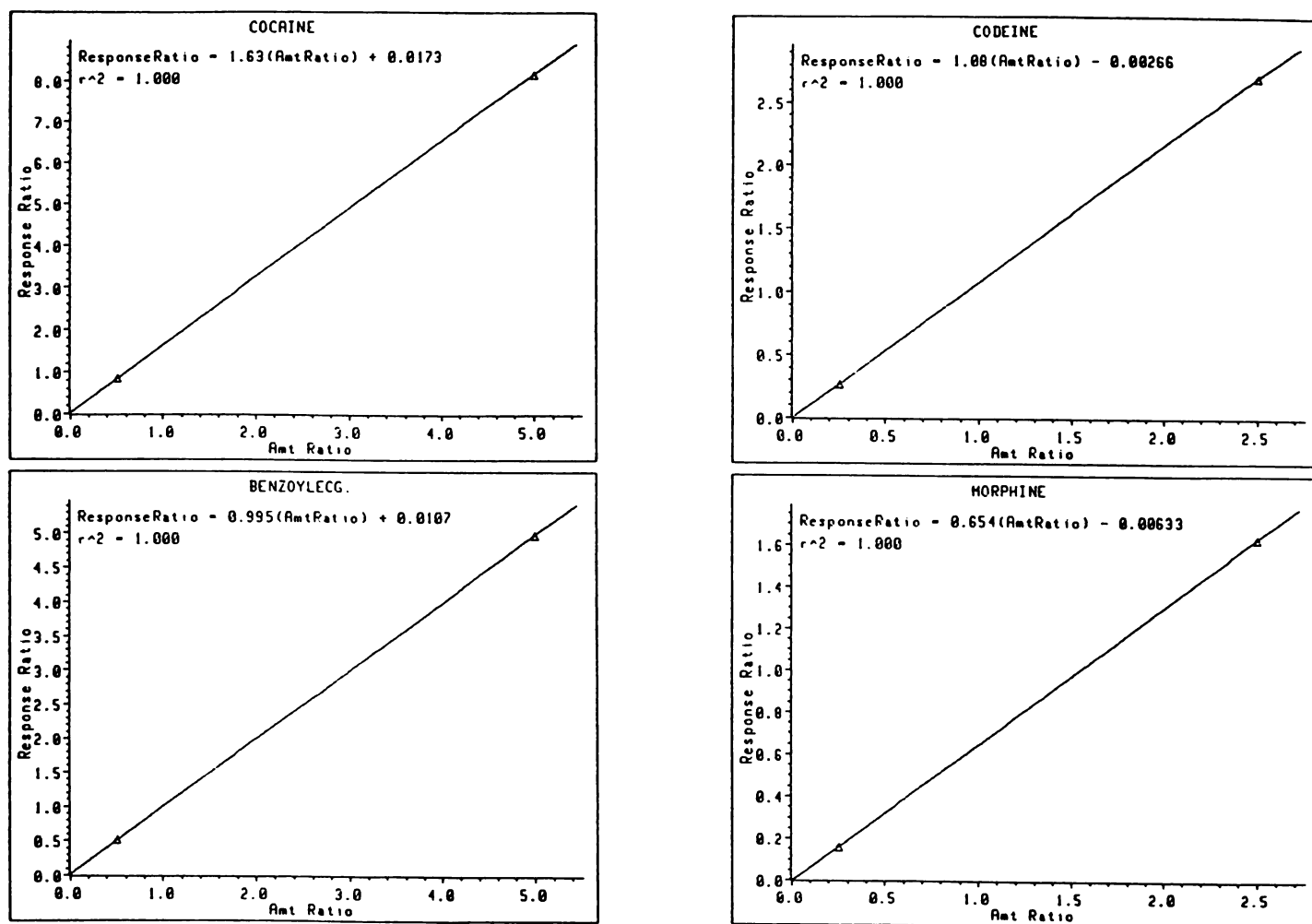


Figure 1. Internal concentration for all four chemicals are 0.20 ug/ml. All four calibration tables are constructed from two points; 0.10 ug/ml, 1.00 ug/ml with the origin included. Response ratio = area of drug/area of internal standard. Amount ratio = concentration of drug/concentration of internal standard.

Application of the Milenia Cocaine Metabolite Kinetic Enzyme Immunoassay to Screening Whole Blood Samples

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Beginning in the early 1970s there has been an ongoing interest in modifying or adapting commercial enzyme immunoassays for the purpose of rapidly screening for drugs of abuse in whole blood or tissue samples. Several of these adaptations have been successful, but resulted in a very tedious and time-consuming analysis, others have somewhat solved this problem but still not attained the speed that is needed in current forensic toxicology analyses.

The objective of this study was to adapt a commercially available kinetic enzyme immunoassay (EIA) to screening whole blood (DUID and post mortem specimens) for cocaine, benzoylecgonine, and cocaine metabolites. The project utilized the Milenia Kinetic EIA System manufactured by Diagnostic Products Corporation (DPC), Los Angeles, CA, and the Milenia Cocaine Metabolite assay likewise by DPC.

Calibrators were made by spiking blood bank blood with benzoylecgonine and the sample volume was increased to 50 μ L. The blank response (mOD/min) for blood bank blood was found to be not significantly different from that for negative postmortem blood or negative antemortem blood collected on NaF/Oxalate from laboratory volunteers. The blank mean was 206.4 mOD/min, SD = 7.4 mOD/min, CV = 3.6%. The limit of detection determined as the mean blank response minus 3 SD was 10 ng/ml.

From the blood calibration curve a cutoff concentration for positives of 50 ng/ml was selected. The precision at the cutoff was a mean of 159.6 mOD/min, SD = 4.2, CV = 2.6%. The difference between the negative blood reference and the cutoff reference was 46 mOD/min or greater than 10 SDs.

Interindividual variation for negative specimens collected from 15 normal individuals gave a mean of 336 in mOD/min with a standard deviation (SD) of 44. There was no overlap at 3 SD with the cutoff response. Thirty DUID blood samples were analyzed by RIA (cutoff 100 ng/ml) and by the modified EIA (cutoff 50 ng/ml) and by GC/MS (cutoff 50 ng/ml). The 3 methods agreed in 11 positive samples and 15 negative samples with 4 samples being positive by EIA, but negative by RIA and GC/MS. These "false positives" by EIA were attributed to its lower detection limit and its cross-reactivity for cocaine-like compounds (*i.e.*, benzoylecgonine 100%, cocaine 153%, ethylcocaine 60%, and ecgonine methyl ester not detected at 100,000 ng/ml).

In conclusion, we have demonstrated a new modification of a commercially available EIA kinetic system that utilizes whole blood to test for cocaine and cocaine metabolites with a sensitivity of 50 ng/ml. This assay procedure allows for the testing to be accomplished for 40 samples in duplicate for a cost of \$1.25 per sample and throughput of 40 samples in 2 hours. Additionally, this nonisotopic assay presents no problem with waste disposal.

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Identification of Volatile Poisons by Capillary Column Headspace Gas Chromatography/Mass Spectrometry: A Methylchloroform Fatality

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For identifying volatile poisons, capillary column headspace gas chromatography/mass spectrometry (CC HS GC/MS) enjoys three advantages. Capillary columns provide higher sensitivity and resolution than packed columns. Headspace sampling provides cleaner samples than either direct injection or injection of solvent extracts. Mass spectrometry provides specificity unequaled by retention time alone. A suicide by ingestion of automotive brake cleaner containing methylchloroform (MC) illustrates the usefulness of the CC HS GC/MS technique.

MATERIALS AND METHODS

Ethanol, MC, and 2,2,2-trichloroethanol (TCE, a metabolite of MC) were screened and quantitated by HS GC. A Sigma 2000 gas chromatograph/HS-100 headspace sampler (Perkin Elmer, Norwalk, CT) equipped with a flame ionization detector and a 6-foot x 2-mm inside diameter (ID) glass column packed with .2% Carbowax 1500 on 80/100 Carbopack (Supelco, Bellefonte, PA) was used. Column temperature was 120°C 0 min; 10°C/min; 170°C 5 min. Helium carrier gas flow was 20 mL/min., injector and detector temperatures 220°C. Aqueous working standards of ethanol (.025 - .200% w/v), MC and TCE (25 - 200 mg/L) were prepared from stock solutions of ethanol (100 g/L aq), MC (10 g/L methanolic), and TCE (10 g/L aq). To each 20 mL HS vial containing internal standard (1 mL .2% aq n-propanol) and sample (1 mL fluid or 2 g 1:1 aq tissue homogenate) was added 3 g

solid calcium chloride immediately prior to capping and vortex mixing. GC analysis followed a 20-minute equilibration at 70°C.

MC was confirmed in all positive specimens (separate vial, no internal standard) by CC HS GC/MS on a Finnigan Model 4500 GC/MS (Finnigan MAT, Sunnyvale, CA). A 15-m DB-5 capillary column, .25 mm ID and .25 µm film thickness (J & W Scientific, Folsom, CA) was used. Column temperature was 40°C 1 min; 4°C/min; 68°C 0 min. Helium carrier gas linear velocity was 50 cm/sec., injector and manifold were at 250°C. Splitless injection of 20 µL HS followed a 10-minute equilibration at 70°C. Mass/charge was scanned once each second from 40 - 200 Daltons.

RESULTS AND DISCUSSION

Table 1 shows that the higher a volatile's boiling point, the more effectively CaCl_2 salts it out. The very insoluble MC is an exception; apparently MC is so hydrophobic that little salting out can occur. Table 2 shows low to mid-lethal MC concentrations (Baselt and Cravey 1989), probably due mostly to poor specimen packaging although early decomposition (noted at autopsy) may play a role. Excluding stomach content, the fact that the MC level is highest in brain (*i.e.*, MC's lipophilic nature) merely underscores MC's hydrophobic nature. The 1,1-dichloroethane found is most likely a manufacturing impurity (Merck 1989) rather than a metabolite (Baselt and Cravey 1989). Ethanol and TCE

were undetected except for .04% w/v ethanol in stomach content. No other drugs were detected (blood and urine analyzed).

Using gaseous MC standards, acceptable mass spectra were obtained with as little as 4.4 ng MC injected.

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Table 1. Increases in the Concentrations of Volatiles in the Headspace Due to Addition of Different Amounts of Calcium Chloride (Temperature - 70°C).				
Volatile Substance	Boiling Point °C (1)	Solubility 1 Part/ ? Parts H ₂ O (1)	Relative Headspace Concentration (%) (2)	
			1 g CaCl ₂	3 g CaCl ₂
Ethanol	78.5	Miscible	150	142
N-Propanol	97.2	Miscible	172	192
MC	74.1	Insoluble	105	107
TCE	151 - 153	12	297	753
(1) Data from Merck Index, 11th edition (1989).				
(2) No CaCl ₂ added = 100%				

Table 2. 1,1,1-Trichloroethane (MC) Concentrations in a Fatality. mg/L (Fluids) or mg/kg (Stomach Content and Tissues).			
Specimen	Concentration Found	Concentrations in Other Fatalities (6)	
		Average	Range
Blood	14	126	1.5 - 720
Bile (5)	0	---	---
Brain (3), (4)	101	277	3.2 - 590
Kidney (5)	6	56	2.6 - 120
Liver (3), (4)	148	102	4.9 - 220
Lung (4)	9	14	1.8 - 31
Urine (4)	0	1.6	.9 - 3
Stomach Content (4)	104 (7)	---	---
(3) 1,1-Dichloroethane Present (4) Specimen Received Loosely Sealed - No Leaking Fluid (5) Specimen Received Loosely Sealed - Fluid Spill Noted (6) Baselt and Cravey, 1989 (7) 2.8 mg MC in the 27.4 g Submitted			

The Simultaneous Identification of Diazolo and Triazolo-Benzodiazepines in Plasma Samples by GC/MS Using Negative Chemical Ionization

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The specific identification of the diazolo, and recently the triazolo-benzodiazepines (BZD) have presented a unique analytical challenge for the forensic toxicologist. Negative ion chemical ionization mass spectrometry has been used with some success in this challenge (Min and Garland 1977; Miwa and Garland 1981). We have developed a novel method using a capillary column coupled with electron capture negative ion chemical ionization mass spectrometry. This method allows for simultaneous confirmation of oxazepam, diazepam, clonazepam, alprazolam, adinazolam, triazolam, and N-desmethyldiazepam.

Administrative samples were screened using a modified Abbott TDx assay with a cutoff level of 2.5 ng/mL. The positive samples were then quantified for the above BZDs with a cutoff of 2.5 ng/mL by GC-ECD. Then randomly selected GC positive samples were then analyzed by the method described here. Briefly, 0.25 mL of plasma was extracted with 1.5% isoamyl alcohol in toluene. The organic extract was dried under nitrogen, reconstituted in toluene and analyzed using a Hewlett-Packard 5989A GC/MS with methane as the reagent gas. SPB-5 fused silica capillary column (0.25 mm, i.d., 0.25 micron film thickness)

was cut to 15 m in length, so each analysis can be completed within 9 minutes. Helium was the carrier gas. The injector (250°C) was in splitless mode with a purge time of 0.25 minute. The oven temperature began at 150°C with a hold time of 1 minute and ramped at 20°C per minute to 300°C with a final hold time of 1.5 minutes. The ion source and the quadrupole were maintained at 200°C and 100°C, respectively. BZD standards were analyzed under full scan mode to determine the optimum ions for monitoring. For the samples, the instrument was operated in the NCI selective ion monitoring (SIM) mode.

The retention times and ions monitored are listed in Table 1. This method does not require a derivatization step, which eliminates many of the problems involved with specific BZD identifications. All 8 BZDs were identified in the plasma samples using this method, with detection limits of approximately 1 ng/mL. Under the experimental conditions, the most predominant ions of these BZDs were the molecular ions (M), with a few exceptions. Hydroxylated BZD, such as oxazepam and lorazepam produced (M-H₂O) as the predominant ion, which might be due to the thermal instability of the BZDs on the GC. With triazolam, the intensity of the M (m/z 342) was much less than the (M-Cl) (m/z 306).

We have demonstrated that by taking advantage of the separation power of capillary column, coupled with the negative chemical ionization mass spectrometry, we could detect numbers of BZD simultaneously instead of monitoring one or a few BZD(s) by packed column NCI (Rubio *et al.* 1982). This method requires minimal sample preparation, and the GC/NCI MS analysis was rapid and capable of detecting low levels of BZDs in 0.25 mL of plasma.

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Table 1. Retention Times and Ions Monitored for NCI SIM		
Compound Name	Retention Time (min)	Ion Monitored (m/z)
Oxazepam	5.61	268
Lorazepam	5.96	302
Diazepam	6.08	284
Clonazepam	7.67	315
Alprazolam	8.08	308
Adinazolam	8.34	351
Triazolam	8.44	306
N-Desmethyadinazolam	8.61	337

The Analysis for LSD, ISO-LSD, and N-Demethyl-LSD in Body Fluids by Gas Chromatography/Tandem Mass Spectrometry

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Contrary to a widespread public perception that use of LSD is no longer a problem, there is considerable evidence that the illicit drug continues to be used, and in some segments of the population, its use is increasing (Seligmann *et al.* 1992). Factors that have contributed to its continued use are wide availability, low cost, and the difficulty of detecting LSD use by analysis of body fluids. The usual oral dose of LSD is only 20 to 80 micrograms, and the drug is extensively metabolized, primarily to as yet unidentified metabolites. Consequently, conclusive identification of LSD in body fluids from a recent LSD user requires an extremely sensitive and specific analytical method.

Several GC/MS assays for LSD in physiological specimens have been reported (Francom *et al.* 1988; Lim *et al.* 1988, Paul *et al.* 1990), but they lack the sensitivity and specificity needed to consistently confirm LSD in RIA-positive samples. However, a recently developed procedure based on gas chromatography and tandem mass spectrometry (GC/MS/MS) offers dramatically improved sensitivity and specificity for analysis of LSD, iso-LSD (a diastereoisomer often present in illicit samples of the LSD), and N-demethyl-LSD (a metabolite of LSD) in either blood or urine specimens.

MATERIALS AND METHODS

The GC/MS/MS analyses were performed on a Finnigan MAT TSQ-70 triple-quadrupole mass spectrometer system fitted with a 12 m Hewlett-Packard Ultra-2 capillary column. For analysis of urine

specimens, a one-step liquid/liquid extraction was employed. The internal standard, lysergic acid methylpropylamide (LAMPA), was added to 4 mL of each sample of urine. The samples were made basic and extracted with toluene:methylene chloride (7:3 v/v), followed by derivatization with bis-(trimethylsilyl)acetamide (BSTFA). The derivatized extracts were injected (splitless) and the GC oven temperature programmed from 175°C to 298°C. The trimethylsilylated analytes and internal standard were ionized by ammonia positive-ion chemical ionization and only the protonated molecules allowed to pass through the first mass analyzer. The protonated molecules were then caused to undergo collision-induced dissociation to daughter ions, and the ion currents corresponding to structurally diagnostic daughter ions were monitored.

Blood specimens were analyzed similarly, except that an additional solid-phase extraction was included in order to reduce the amount of nonvolatile material injected into the gas chromatographic column.

RESULTS

The mass spectrum produced by collision-induced dissociation (CID) of the protonated molecule (MH^+) of the trimethylsilyl derivative of LSD is shown in Figure 1. The ion current profiles from GC/MS/MS analysis of a urine extract containing 40 pg/mL of LSD, iso-LSD, and the LAMPA internal standard are shown in Figure 2. The limit of detection for LSD and iso-LSD in urine is 10 pg/mL. The limit of quantitation for LSD, calculated as the mean of the background in the

m/z 295 ion current profile plus 10 standard deviations, was determined to be 20 pg/mL. During the analysis of 30 externally-submitted quality control samples over a 2-week period, the ion ratios corresponding to the daughter-ion peak area ratios m/z 280/295 and 353/295 for LSD-TMS and m/z 353/280 of iso-LSD-TMS varied by less than $\pm 20\%$ from the corresponding ratios obtained on a reference standard. Analysis of drug-free urine specimens showed no detectable peaks in the ion current profiles at the retention times for LSD, iso-LSD, or LAMPA.

When positive ion chemical ionization is used, the sensitivity for N-demethyl-LSD-TMS is less sensitive than the analysis for LSD-TMS. However, a limit of detection for N-demethyl-LSD of less than 10 pg/mL can be achieved by conversion of the metabolite to a bis-(trifluoroacetyl) derivative and collision-induced dissociation of the abundant molecular anion (M^-).

ACKNOWLEDGMENTS

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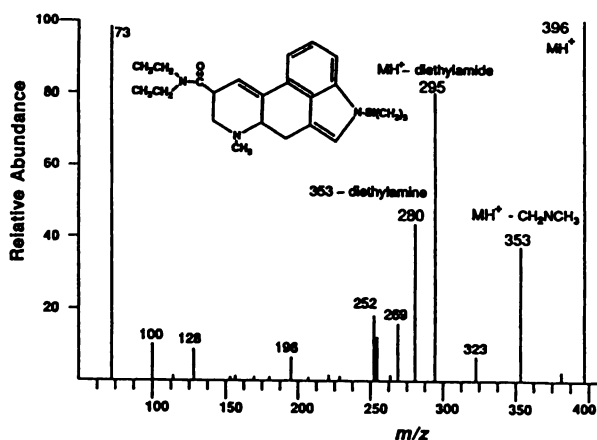


Figure 1. The CID spectrum of the protonated molecule of LSD-TMS (MH^+).

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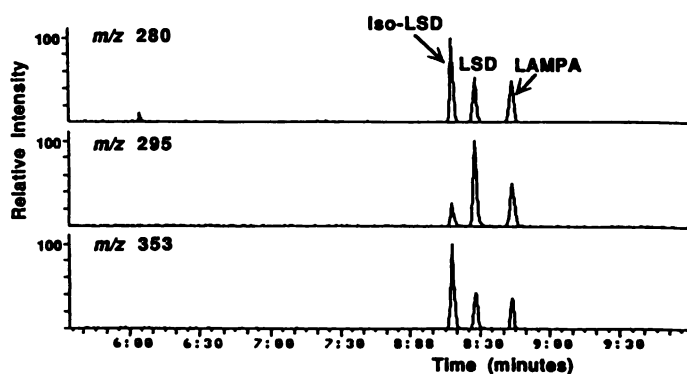


Figure 2. GC/MS/MS analysis of a urine extract containing 400 pg/mL each of LSD, iso-LSD, and LAMPA (the internal standard).

The Use of Tandem Mass Spectrometry (MS/MS) for the Analysis of Forensic Tissue Samples

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The California Department of Justice Toxicology Laboratory currently analyzes over 5,000 tissue samples annually for the presence of drugs and their metabolites. These samples are obtained from drivers in the state of California suspected of driving under the influence of drugs and/or alcohol. In order to more efficiently utilize available resources, this laboratory has employed a MS/MS to detect a number of drugs and their metabolites. This paper summarizes the details and advantages of the use of MS/MS over traditional single quadrupole analyzers and summarizes the drugs that are detected by this method in this laboratory.

Single stage mass spectrometers detect drugs with a single ionization either by electron impact or chemical ionization followed by filtering by a single quadrupole. A MS/MS employs these same principles; however, sensitivity and specificity are enhanced by the addition of a reaction chamber and a second set of quadrupole rods. In a typical MS/MS analysis the first quadrupole (Q1) is set up to transmit a single ion which was created in the ion source. This ion, or "parent ion", then enters the reaction chamber (Q2) where a combination of argon and electronic energy cause the formation of "daughter ions." The second quadrupole (Q3) then transmits only the appropriate daughter ion which is then detected by a photon multiplier. This method is also called multiple reaction monitoring. The increase in sensitivity and selectivity allows the use of smaller sample volumes and simpler extraction schemes.

Samples to be analyzed by MS/MS are extracted using a one step liquid-liquid extraction. A small amount of sample (0.2 - 2 milliliters) is extracted with

ethyl acetate (Fisher Scientific) at a pH optimized for maximum extraction efficiency. Deuterated analogs of the analyte(s) are used as internal standards. The extract is then evaporated and derivatized with pentafluoropropanol (PFP) and/or pentafluoropropionic anhydride (PFPA) (Pfaltz and Bauer, Inc). The samples are then analyzed on a model Trio 3 tandem mass spectrometer (VG Masslab) monitoring the appropriate parent to daughter ion transition(s). The appropriate transitions are determined by a series of experiments that utilize the solid probe attachment of the analyzer. Small amounts of dried, derivatized standards are placed on the probe and inserted into the ion source. The analyzer's data system is then programmed to monitor selected ions in both Q1 and Q3 while argon gas pressure and collision energy are optimized for maximum ion transmission.

This laboratory analyzes for two types of drugs by MS/MS, opiates and cocaine. Opiate analysis employs electron impact ionization followed by the detection of various daughter ions. The opiates currently analyzed are morphine, 6-monoacetylmorphine, and codeine. The analysis of hydrocodone and oxycodone is currently under development. The opiates are extracted at a pH of approximately 9.5 and derivatized with PFPA. Cocaine and its metabolites benzoylecgonine and ecgonine methyl ester are simultaneously detected by utilizing positive chemical ionization and multiple reaction monitoring. Extraction of these compounds is at pH 8 with derivatization by PFPA and PFP.

In the future this laboratory is investigating the use of MS/MS for the analysis of other drugs including cannabinoids, ethylbenzoylecgonine (cocaethylene).

Sudden Unexpected In Utero Fetal Death in Association with Cocaine Exposure: Case Presentations and a Review of the Literature

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Cocaine abuse by pregnant women has become a significant public health problem and presents as a medical crisis for both the mother and fetus. In Onondaga County crack replaced cocaine hydrochloride abuse resulting in a dramatic increase in ER visits and fetal deaths. The purity of street cocaine (HCl) went from about 18% - 30% to 80% - 90% pure crack. The use of illicit and misused drugs is common among pregnant women regardless of race or socioeconomic status. A number of studies have reported correlations of in utero cocaine exposure to adverse neurological consequences. The use of cocaine during the last trimester of pregnancy has been associated with fetal distress, premature labor, abruptio placenta, and still births.

The Maternal-Placental-Fetal unit provides unique problems in understanding cocaine toxicity to the fetus. Most of what is clearly understood regarding the pharmacokinetics and pharmacodynamics of cocaine in the pregnant female results from experimental animal data and limited human case documentation.

This report outlines five cases from our recent crack epidemic and serves to highlight numerous publications of similar findings from across the nation. This selection of literature not only covers basic physiology and pharmacology of the human fetus, but also covers relevant animal models.

METHODS

Blood, urine, meconium, and/or other tissues were collected from all autopsied fetuses. The blood samples

were preserved with 2% sodium fluoride. Tissues were frozen until analysis. Blood and tissue homogenates were protein precipitated using acetone.

Urine, modified blood, and tissues were screened by immunoassay using SYVA DAU kits. The meconium extraction technique is outlined in Figure 1. Positive cocaine screens were confirmed by GC/MS, utilizing solvent extraction, derivatization, and deuterated IS.

Identification and quantitation was performed by select ion monitoring following electron impact ionization using a HP 5970 mass selective detector coupled to a high resolution capillary column gas chromatograph.

RESULTS

Maternal histories, fetal pathology, and toxicological results are summarized in Table 1. The histories were abstracted from maternal/neonatal medical records. Table 2 is the toxicology summary for case # 5. Tables 3, 4, and 5 are the results of 32 cases from directed assays of paired urine and meconium samples obtained from a local hospital neonatal ICU. Table 6 is data from five of the directed assay cases resulting from multiple urine collection.

DISCUSSION AND CONCLUSIONS

Our most common finding was abruptio placenta with meconium staining. The mothers were cigarette smokers having little or no prenatal care. There is a strong correlation between maternal age, number of

pregnancies, and the severity of cocaine abuse. Maternal history is an unreliable guide to the investigation of fetal death.

Autopsies performed on fetal remains offered little information as to the cause of death. Cocaethylene is more potent than cocaine in mediating lethality and probably contributed to the death of the full-term fetus in case 5. All deaths were certified as fetal or neonatal demise in association with maternal cocaine use.

It has been suggested that meconium is a better specimen for screening neonates for in utero drug exposure. Data from our 32 cases support this idea. Meconium analysis is not as readily available to the clinical lab nor is it as easy, inexpensive, or as rapid as urine drug screening. Meconium does offer an alternative to urine drug testing the neonate. Meconium

formation begins in the 16th week of pregnancy and thus would represent drug exposure until birth. Our barbiturate and opiate positive samples had documented maternal administration the night before or day of delivery. The cutoff concentration utilized by some immunoassays for cocaine metabolite is not sufficiently sensitive to detect casual drug exposure in the neonate.

Calibration of the cocaine assay using 75 ng/ml urine calibrator will increase the detection of low level cocaine concentrations most often associated with fetal drug exposures.

Data presented in Table 6 indicate acute exposure cases and, by utilizing the lower cutoff, detection times approach those seen with recreational cocaine use in adults.

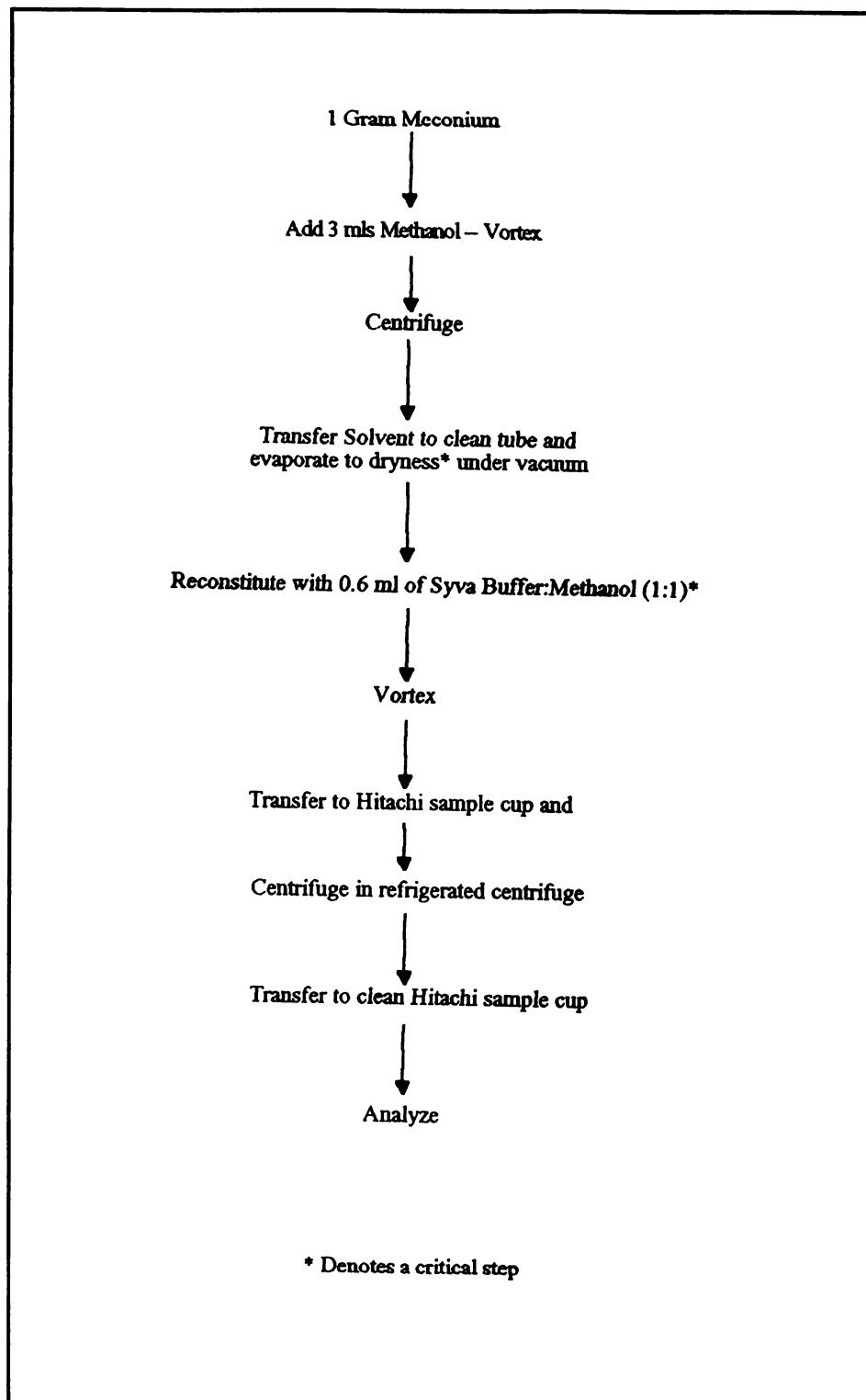


Figure 1. Meconium Flow Chart.

Table 1. Summary of Cocaine Associated Fetal Deaths		
Maternal History	Fetal Pathology	Toxicology Results
<p>Case #1</p> <p>20-year-old, no prenatal care. Hx of ethanol and speed abuse. Two weeks since last drug exposure. Ruptured membranes 12/10.</p> <p>G₁P₀ smoker.</p>	<p>30 weeks gestation.</p> <p>Autolysis.</p> <p>1300 grams, 25 cm HC</p> <p>Delivery 12/16.</p>	<p>No drugs detected.</p>
<p>Case #2</p> <p>35-year-old, 10-year hx cocaine. Last cocaine 12/13, admission on 12/14.</p> <p>G₄P₂₀₄₂ smoker.</p>	<p>20 weeks gestation.</p> <p>250 grams, 16 cm HC.</p> <p>Macerated fetus.</p> <p>Abruptio placenta.</p>	<p>Tissue only.</p> <p>Liver 2.3 mg/kg benzoylecgonine.</p> <p>No other drugs detected.</p>
<p>Case #3</p> <p>28-year-old. No hx of drugs. Chronic hypertension and sexually transmitted diseases. Admitted 1/18.</p> <p>G₄P₂₀₁₂</p>	<p>37+ weeks gestation.</p> <p>28 cm HC.</p> <p>Term fetus delivered 1/22.</p> <p>Abruptio placenta.</p>	<p>Brain and meconium.</p> <p>+ Cocaine</p> <p>+ Ecgonine methylester</p> <p>+ Benzoylecgonine</p>
<p>Case #4</p> <p>23-year-old, used cocaine early.</p> <p>Meconium staining.</p> <p>G₃P₁₀₁₁</p>	<p>2-day-old female.</p> <p>26 - 27 weeks gestation.</p> <p>Intercranial bleed.</p> <p>919 grams.</p>	<p>Brain 1.1 mg/kg.</p> <p>Benzoylecgonine</p> <p>Meconium + benzoylecgonine</p>
<p>Case #5</p> <p>28-year-old, used cocaine on delivery.</p> <p>No prenatal care.</p> <p>Last fetal movement day before delivery.</p> <p>G₇P₅₁₁₃</p>	<p>Term fetus, 3790 g.</p> <p>Still born.</p> <p>Abruptio Placenta.</p> <p>Meconium staining.</p> <p>36 cm HC</p>	<p>Heart BAC 0.03 g%.</p> <p>Ocular fluid ethanol 0.03 g%.</p> <p>See Table 2.</p>
<p>G = Gravida</p> <p>P = Para (Term, premie, abortions, living)</p> <p>HC = Head Circumference</p>		

Table 2. Toxicological Findings for Case #5.		
Specimen Type	Drug Detected	Results
Heart Blood	Cocaine Ecgonine methylester Cocaethylene Benzoyllecgonine Ethanol	0.14 mg/l ~0.1 mg/l <0.1 mg/l 2.5 mg/l 0.03 g%
CSF (Cerebro-Spinal Fluid)	Cocaine Cocaethylene	Trace Trace ¹
Ocular Fluid	Cocaine Ethanol	QNS ² 0.03 g%
Meconium (Postmortem)	Benzoyllecgonine	Positive
Brain	No Drugs Detected	
Urine	Cocaine Ecgonine methylester Ecgonine ethylester Cocaethylene Benzoyllecgonine	Positive Positive Positive Positive Positive
¹ - Less than 0.05 mg/l.		
² - Quantity Not Sufficient for Analysis.		

Table 3. Results of Paired Meconium and Urine Analyses From Selected Cases.			
Case Number	Meconium Result	Urine Result	Reason for Directed Assay
1	Barbiturate	Barbiturate	Late Care Seeker
2	Barbiturate	Barbiturate	Late Care Seeker
3	None Detected	Opiate	Late Care Seeker
4	None Detected	Barbiturate	History of ETOH
5	Fecal Interference	Barbiturate	Late Care Seeker
6	Barbiturate	Barbiturate	Late Care Seeker
7	Amphetamine	None Detected	No History
8	None Detected	Barbiturate	Late Care Seeker

Table 4. Results of Paired Meconium and Urine Analyses From Selected Cases.			
Case Number	Meconium Result	Urine Result	Reason for Directed Assay
9	Cannabinoids	None Detected	History of ETOH, Marijuana
10	Cannabinoids	Cannabinoids	History of ETOH, Cocaine
11	Cannabinoids	None Detected	Possible Drug Use
12	Cannabinoids	Cannabinoids	History of ETOH, Drugs
13	Cannabinoids	None Detected	History of ETOH
14	Cannabinoids	Cannabinoids	History of ETOH, Marijuana
15	Cannabinoids	(a) Amphetamine (b) None Detected	History of Drugs
16	Cannabinoids	None Detected	History of Cocaine, Denies Pregnancy
17	Cannabinoids	Cannabinoids	Late Care Seeker
18	Cannabinoids	None Detected	History of ETOH, Drugs
19	Cannabinoids	Cannabinoids	History of Drugs
20	Cannabinoids	None Detected	ETOH Abuse

Table 5. Results of Paired Meconium and Urine Analyses From Selected Cases.			
Case Number	Meconium Result	Urine Result	Reason for Directed Assay
21	Benzoylecgonine	Benzoylecgonine	Chlamydia, Late Care Seeker
22	Benzoylecgonine	Benzoylecgonine	No Prenatal Care
23	Benzoylecgonine	Benzoylecgonine	Possible Drug User
24	Benzoylecgonine	Benzoylecgonine	Late Care Seeker, Previously (+) Cocaine
25	Benzoylecgonine	Benzoylecgonine	Late Care Seeker, History of Cocaine
26	Benzoylecgonine	Benzoylecgonine	History of Cocaine
27	Benzoylecgonine	None Detected	Late Care Seeker
28	Benzoylecgonine	None Detected	History of Cocaine
29	Benzoylecgonine	Benzoylecgonine	History of Drugs
30	Benzoylecgonine	Benzoylecgonine	Substance Abuse
31	Benzoylecgonine	None Detected	Late Care Seeker
32	Benzoylecgonine	Benzoylecgonine	ETOH, Marijuana, No Prenatal Care

Table 6. Post-Partum Duration of Benzoylecgonine in Newborns.					
Case Number	Date and Time of Birth	Urine Sample Date/Time	EIA Result	EIA Value	Confirmation
#22	01/28 11:16	01/29 18:00	Benzoylecgonine	110	Benzoylecgonine
		01/30 17:30	Benzoylecgonine	77	Not Done
		01/31 08:35	Benzoylecgonine	52	Not Done
		02/01 06:00	Benzoylecgonine	47	Not Done
		02/01 09:00	Benzoylecgonine	27	Not Done
#23	05/02 08:31	05/04 00:30	Benzoylecgonine	36	Benzoylecgonine
		05/05 12:00	Benzoylecgonine	26	Not Done
		No Date/Time	Benzoylecgonine	9	Not Done
#24	07/22 09:40	07/23 20:00	Benzoylecgonine	62	Benzoylecgonine
		07/24 14:35	Benzoylecgonine	18	Not Done
		07/24 19:15	Benzoylecgonine	-1	Not Done
		07/25 04:30	Benzoylecgonine	-22	Not Done
		07/26 08:30	None Detected	-33	Not Done
#26	11/09 05:50	11/09 14:15	Benzoylecgonine	74	Benzoylecgonine
		11/13 15:45	None Detected	-32	Not Done
#29	12/18 17:44	12/19 14:15	Benzoylecgonine	66	Benzoylecgonine
		12/21 11:00	Benzoylecgonine	51	Not Done
Note: EIA values are relative to a SYVA 300 ng/ml benzoylecgonine calibrator which yields a value of zero when tested in the same manner as patient specimens. Negative control samples gave readings of -34 (±5). All assays were performed on a Hitachi 705 analyzer.					

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Catha Edulis (KHAT): New Problems on the Clandestine Market of Drugs in Italy

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Celastrus edulis is a plant belonging to the Celastraceae family and native in the countries of the Eastern Coast of Africa. The Catha edulis is a slender, straight, short tree reaching a height of four meters, with persistent oval, usually opposite finely toothed leathery leaves. The flowers are small and white and are grouped into axillary buds. The fruit is an elongated or club-shaped capsule containing between one and three seeds.

In recent years, the flow of immigrants to Italy from African countries has increased. Most of the immigrants come from Somalia in increasing numbers, as a result of past cultural and trading ties and also because of the civil war that broke out recently in that country. This situation prompts the spreading among the populations of activities aimed at alleviating the burden of livelihood and of surviving.

In particular, it is reported that the use of "Celastrus edulis" or "Catha edulis", a tree that has grown spontaneously in East Africa for centuries, has spread from its native countries (namely Somalia, Kenya,

and Ethiopia) to Sudan and to the countries of the Middle East such as Yemen and Saudi Arabia.

The leaves and buds are either chewed or used to make an infusion in much the same way as with tea. Alkaloid substances having effects similar to those produced by narcotics and amphetamine such as Cathine and Cathinone are thus introduced into the body.

In Italy as of January 10, 1988, such alkaloids have been considered as drugs and the maximum amount of Cathine allowed for individual use was established since March 7, 1990, after the act was reviewed. Above this amount its use is to be considered illegal.

CHEMISTRY OF KHAT OR CIAT (CELASTRUS EULIS)

Different studies carried out by a number of researchers on Catha edulis have made it possible to identify many of its active products, but not all of them. The active principles, phenylalkylamines, present in

larger amounts and identified only recently with accuracy, are:

Cathine [(+)-norpseudoephedrine]

Cathinone [(-)-alpha-aminepropiofenone]

There are many others, but the stimulant effect of this plant appears to be due to the presence of the above-mentioned active principles.

Cathinone is suspected to be the precursor of Cathine, but the mechanism whereby it is transformed is not yet clear. It has been observed experimentally that in the fresh leaves the main component is Cathinone and Cathine is usually less than 0.2%, whereas in the dried product the main component is Cathine.

TEST METHOD (EXTRACTION PROCEDURE)

Five grams of finely chopped fresh leaves and stalks were treated with 100 ml of HC10.1 N. After filtering, the acid solution was transferred to a Squibb separation funnel and the extract was made alkaline with 1 N sodium hydroxide and extracted with two 50 ml portions of trichloromethane. The organic extracts were collected, dehydrated, filtered, and evaporated to dryness using a nitrogen vacuum rotary evaporator. The residue was

dissolved with an adequate *volume* of trichloromethane and analyzed in the GLC/MS.

TEST METHOD (GLC/MS ANALYSIS)

A Hewlett-Packard 5890 gas chromatograph was used, interfaced with a mass spectrometer (HP-5970 system). The separation column used was a 25-meter-long Hewlett-Packard ULTRA-2 (5%-phenylmethyl silicone-SE54), with an inner diameter of 0.2 mm and with a stationary phase film 0.33 μ m thick directly interfacing the mass spectrometer. The working conditions were as follows:

Carrier (He): linear velocity u at 70°C of 26.4 cm/sec for a flow of 0.5 ml/minute.

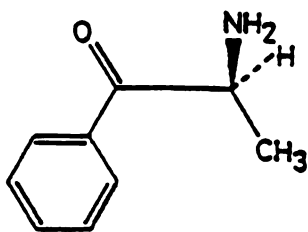
Injection method: splitless

Injector temperature: 250°C

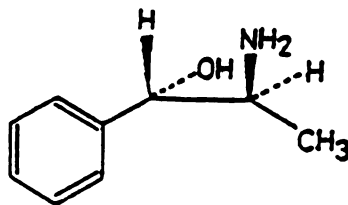
Temperature plan: 70°C for 3 minutes; 25°C/minute up to 145°C; 10°C/minute up to 280°C

Temperature transfer line GLC/MS: 250°C

Source temperature MS: 200°C



cathinone



(+) - norpseudoephedrine

KATHA EBULIS' extract 5 grams/10 ml - 1 ul injected

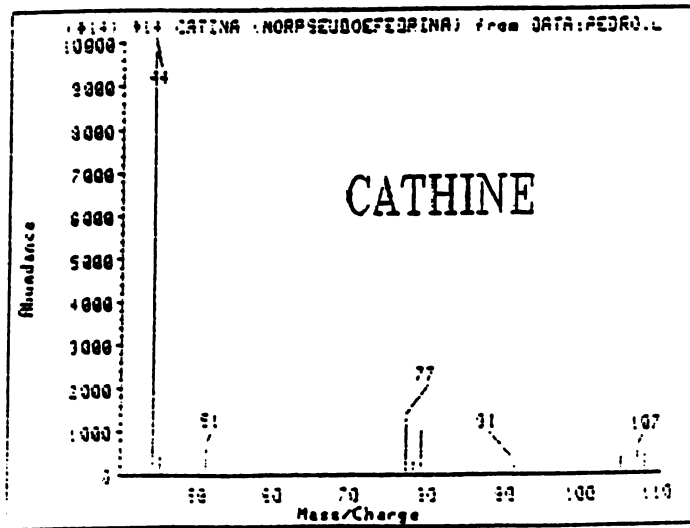
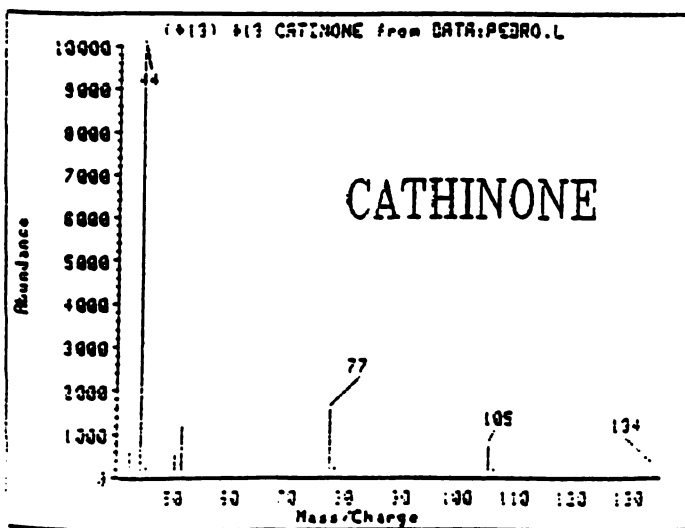
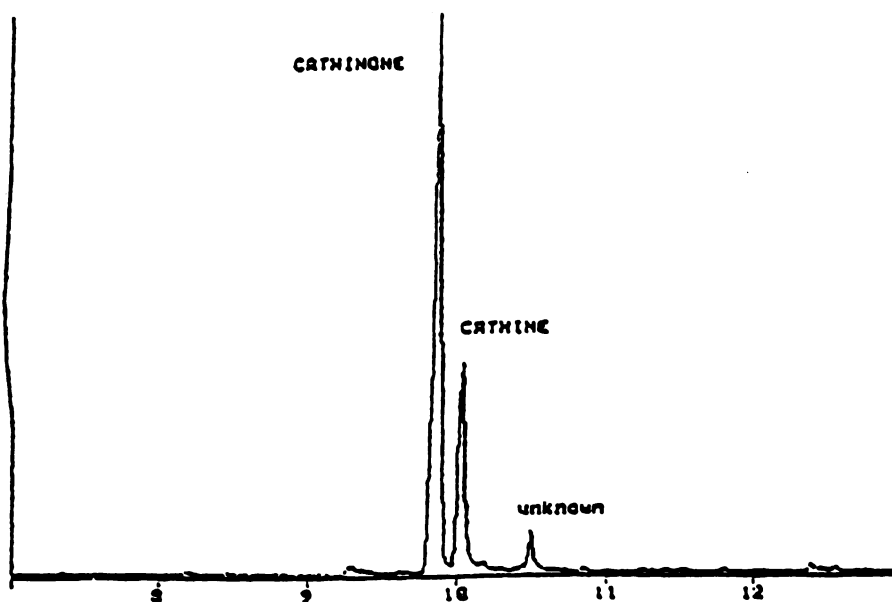


Figure 1.

The Use of Clotted Blood for the Determination of Carboxyhemoglobin: A Comparison to Unclogged Whole Blood Values

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Clotted blood has been suggested as a useable specimen for the analysis of carbon monoxide. Frequently clot, but no liquid blood, is available from autopsies, especially with unattended natural deaths. For each case studied, we compared carboxyhemoglobin (COHb) values of clotted to unclotted blood.

EXPERIMENT

Clotted and unclotted forensic blood specimens were prepared with modifications (Freireich and Landau 1971). For each case, volumes of at least 1 milliliter of homogenated clotted blood and unclotted blood were mixed individually by vortexing in a test tube containing 40 - 45 milligrams of sodium hydrosulfite. No further treatment is necessary for the unclotted blood sample. The homogenate was diluted with an equal volume of 10% Triton-X-100 solution, vortexed, and centrifuged for 5 minutes at 3,000 rpm.

Duplicate 100 microliter aliquots of each sample, clotted blood supernatant and unclotted blood, were obtained using capillary pipets.

The analysis was performed by introducing the sample by pipet into a Corning 2500 CO-oximeter (Corning, NY, 1983), bringing it to temperature,

hemolyzing, and measuring the percent saturation of COHb. The values of COHb were recorded for each aliquot.

RESULTS AND DISCUSSION

The specimens used in this study represented two carbon monoxide poisoning deaths and ten randomly selected cases with various causes of death other than selected cases with various carbon monoxide poisoning. Results are shown in Table 1.

This comparison suggests that testing clotted blood for COHb produces a value within 10% of the value for unclotted whole blood. This is sufficiently close for virtually all practical forensic and clinical purposes. Therefore, clotted blood may be an adequate specimen for forensic COHb analyses in absence of unclotted whole blood.

REFERENCES

- Corning. *2500 CO-oximeter Instruction Manual*, 478391 Rev. F, 1983.
- Freireich, A.W. and Landau, D. Carbon Monoxide Determination in Postmortem Clotted Blood, *Journal of Forensic Science* (1971) 16(1):112-119.

Table 1. Average Carboxyhemoglobin Saturation Values and the Percent Difference Between Clotted and Unclogged Blood.

Case	Saturated COHb Clotted Average	Saturated COHb Unclogged Average	Percent Difference of Averages
1	2.30	2.55	9.8
2	61.05	56.55	7.9
3	77.50	74.00	3.6
4	6.00	6.05	0.8
5	2.45	2.45	0
6	2.55	2.75	7.2
7	7.05	7.05	0
8	3.20	3.30	3.0
9	3.85	3.95	2.5
10	2.55	2.70	5.5
11	2.65	2.80	5.3
12	2.95	3.05	3.2

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